

**NERVE GROWTH FACTOR: ITS ROLE IN MALE FERTILITY
AS AN OVULATION INDUCER**

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By

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ABSTRACT

The studies presented in this thesis were designed to elucidate whether the abundance of ovulation-inducing factor/nerve growth factor (OIF/NGF) in alpaca semen can be used as a biomarker to predict male fertility. The neurotrophin, OIF/NGF has been identified in camelid, cattle and human semen. It is only in camelids, however, that an ovulation-inducing role for OIF/NGF has been described. The information gathered from several studies clearly demonstrate that this protein is the stimulus responsible for initiating the ovulatory cascade in camelids. In addition, intramuscular administration of OIF/NGF resulted in a dose-dependent response in terms of ovulation rate, corpus luteum (CL) lifespan, luteinizing hormone (LH) and progesterone secretion. I hypothesized that the quantity of OIF/NGF differs among male alpacas and this abundance arbitrates ovulation and pregnancy rates as well as CL formation and function. To substantiate this hypothesis, the following questions were answered: 1) can OIF/NGF in alpaca semen be quantified using a radioimmunoassay; 2) does the concentration and total abundance of OIF/NGF in alpaca semen vary within and among male ejaculates; 3) what is the glandular source of OIF/NGF that contributes to the male ejaculate; 4) is OIF/NGF concentration or abundance related to parameters associated with male fertility; 5) can OIF/NGF concentration or total abundance in the ejaculate discriminate fertile and subfertile males using both retrospective and prospective approaches; and 6) can power Doppler ultrasonography be used to assess the luteotrophic effect of OIF/NGF in tissue vasculature of the developing CL? I discovered that the source and the amount of OIF/NGF varies among species. In llamas, OIF/NGF is produced by both the corpus and disseminate portions of the prostate gland. In rats, OIF/NGF was detected in testis interstitial cells and in the lumen of the coagulating gland

(anterior prostate). Ovulation-inducing factor/NGF secretion by the ampullae and vesicular glands contributed to its presence in bull (cattle and bison) ejaculates. In elk and white tail deer, OIF/NGF was detected in the ampullae and prostate glands, respectively. To gain an understanding of the abundance of OIF/NGF in ejaculates and changes in its concentration within and among males, OIF/NGF levels in semen were quantified using the radioimmunoassay. The assay developed exhibited parallel displacement curves among recombinant NGF, OIF/NGF purified from llama seminal plasma, llama and bull (cattle) seminal plasma. Ovulation-inducing factor/NGF comprised a greater percentage of the total protein found in camelid ejaculates than in cattle. Ovulation-inducing factor/NGF concentration correlated positively with sperm concentration and negatively with pH and semen volume, while total abundance of OIF/NGF was related to total prostate area and OIF/NGF concentration. Although a correlation was found between sperm concentration, neither OIF/NGF concentration nor total abundance was associated with higher ovulation, pregnancy or live birth rates. A clear association of the quantity of OIF/NGF in the male ejaculate at breeding and CL form and function was not evident. The measurement of CL vasculature by power Doppler ultrasonography, however, was able to determine nonpregnancy in alpacas earlier than the assessment of changes in CL diameter. In summary, my results did not support the hypothesis that the measurement of OIF/NGF concentration or total abundance in alpaca semen can be used to predict fertility in male alpacas.

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DEDICATION

I dedicate my thesis to my family. Mom, Dad and Ev- without your unconditional love, laughter, support and most of all patience, this would not have been possible. To quote the great Tina Turner: *You're simply the best.*

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LIST OF ABBREVIATIONS

ADAMTS-1	A disintegrin and metalloproteinases with thrombospondin motifs 1
ANOVA	Analysis of variance
A	Area
AV	Artificial vagina
BSA	Bovine serum albumin
BDNF	Brain-derived neurotrophic factor
C(#)	Cervical vertebrae
CD34+	Circulating endothelial progenitor cells
CL	Corpus luteum
cpm	Counts per minute
CRISP3	Cysteine-rich secretory protein 3
Da	Dalton
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
° C	Degree Celcius
EGR-1	Early growth response protein- 1
EE	Electroejaculation
eCG	Equine chorionic gonadotrophin
FPLC	Fast protein liquid chromatography
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone

g	Gravitational force
HA	Hydroxyl-apatite column
HCl	Hydrochloric acid
H&E stain	Hematoxylin and eosin stain
h	Hours
HRP	Horseradish peroxidase
hCG	Human chorionic gonadotrophin
IGF1	Insulin-like growth factor 1
im	Intramuscular
iu	Intrauterine
I	Iodine
kDa	Kilodalton
kg	Kilogram
LC	Liquid chromatography
L(#)	Lumbar vertebrae
LH β	Luteinizing hormone β subunit
μ g	Microgram
mL	Milliliter
μ L	Microliter
μ m	Micron
min	Minute
MHz	Megahertz
mRNA	Messenger RNA

M	Molar concentration (Molarity)
MS	Mass spectrometry
ng	Nanogram
NT-4	Neurotrophin-4,
NGF	Nerve growth factor
N	Normal concentration (Normality)
NS	Not significant
n	Population size
OIF/NGF	Ovulation-inducing factor (NGF derived from seminal plasma)
p75	Low-affinity NGF receptor (Papyrus 75)
%	Percent
PBS	Phosphate buffered saline
PC12	Pheochromocytoma 12
π	Pi
PD-column	Prepacked disposable column
PLUNC	Palate, lung, and nasal epithelium
PEG	Polyethylene glycol
pg	Picogram
PGF _{2α}	Prostaglandin F _{2α}
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RNA	Ribonucleic acid

S(#)	Sacral vertebrae
SAS	Statistical analysis system
SD	Standard deviation
SDS-PAGE	Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SP(1 or 2)	Seminal plasma protein (1 or 2)
TrkA	Tropomyosin-related kinase A
T(#)	Thoracic vertebrae
2D	Two dimensional
VEGF	Vascular endothelial growth factor
vs.	Versus
v/v	Volume to volume

1.0 GENERAL INTRODUCTION

The stimulus or manner by which ovulation-induction is initiated are the criteria used to differentiate spontaneous from reflex-induced ovulators [1]. While both mechanisms of ovulation are dependent on the luteinizing hormone (LH) surge, the trigger for LH release is different. Spontaneous ovulation occurs when the negative effect of progesterone is removed and an increase in estradiol levels generate a positive feedback to the hypothalamus causing an immense release of GnRH which then evokes a surge of LH secretion from gonadotrope cells. This cyclic process is observed in human and nonhuman primates [2-5] and most of our domestic animals including cattle, ewe, pigs and horses[6-10]. In reflex ovulators, stimuli associated with mating are responsible for the initiation of the preovulatory cascade. By definition, *reflex ovulation is brought about by afferent impulses from the genitalia, eyes, ears and nose that converge on the ventral hypothalamus and provoke an ovulation-inducing release of LH from the pituitary* [11]. The complexity involved in understanding the physiology of reflex ovulators is caused by the variety of factors associated with breeding and the differential response on ovarian activity among species. For example, in alpacas, intromission was thought to be required for ovulation in females [12]. A more recent study showed that manual stimulation of the female tract did not cause ovulation; abrasion of the endometrial lining and deposition of seminal plasma, however, induced an ovulatory response [13]. Along with intromission, sensory stimulation caused by treading of the male's forelimbs on the female's backside or auditory stimulation from humming sounds emitted by the male were not triggers for ovulation in these animals [12]. In the koala, glass rod stimulation of the cervix was not associated with CL formation nor progesterone secretion [14]. On the other hand, it is common veterinary practice to mechanically induce ovulation in domestic cats displaying signs of estrous [15]; and in

ovariectomized ferrets, stimulation of the vagina and cervix in the presence of a male increased LH levels in circulation [16]. Rabbits and cats appear to be more sensitive to external stimuli for ovulation induction. Does [17] and queens [18] ovulated when housed together and this observation suggests that males do not provide a distinctive stimulus that is required for ovulation.

Several hypotheses have been proposed to explain the evolution or benefit of different reproductive strategies. The most prevalent theory suggests that mating-induced ovulation is the primitive form, and due to various selection pressures, spontaneous ovulation evolved. In support of this hypothesis, induced ovulation is seen in primitive Orders, *Insectivora* and *Rodentia* [1, 19-21], as well as the Infraclass *Marsupialia* which includes the koala and grey short-tailed possum [14, 22, 23]. Different from most nonmammalian species, the red-sided garter snake has also been shown to ovulate with mating [1, 24]. No clear discernible patterns, however, have been established using phylogenetic constructs to shed light on evolutionary trends of ovulation [25, 26]. Instead, it appears that induced ovulation evolved multiple times within individual species which provides an explanation as to why there is not one strict copulatory stimulus conserved to all induced ovulators [27]. The observation that mating, genital stimulation and seminal plasma can influence female physiology within unrelated species, supports Conaway's opinion that *the phenomenon of induced ovulation spans throughout most Orders and should not be considered as an opposition of spontaneous ovulation, but rather both traits should be viewed as two extremes of a single continuum* [28]. This notion was later agreed upon and acknowledged that *all* mammals express *some* degree of induced ovulation [29, 30].

Induced ovulation may be related to major population fluctuations [31, 32]. The synchrony of male and female gametes improves the likelihood of fertilization, thereby

promoting a rapid recovery from low population levels [31, 32]. Animals which display confined or solitary behaviours would also benefit from induced ovulation. For instance, if contact between males and females is scarce and if ovulation occurs following mating, then the chance of pregnancy would then be maximized [31, 33].

A protein that has further complicated our understanding of induced ovulation is the neurotrophin, nerve growth factor (NGF). In 1986, Dr. Rita Levi-Montalcini received the Nobel Prize for the discovery and demonstration of importance of this 26 kDa homodimer protein in nerve development and maintenance [34-37]. Elucidation of its role in ovulation-induction and in camelid reproductive physiology has only begun. In camelids, one of the earliest known studies which alluded to a purpose for seminal plasma beyond sperm transport and capacitation was done approximately 30 years ago. Chen et al., observed that the administration of seminal plasma could elicit an ovulatory response and luteal development in the Bactrian camel [38]. At that time, it was unknown that this ovulation-inducing factor (OIF) driving this phenomenon was β -nerve growth factor (NGF)[39, 40]. For clarity, β -NGF of seminal plasma origin will be referred to as OIF/NGF throughout the thesis. Thus far, OIF/NGF has been detected in the ejaculate of camelids [39-41], cattle [42-44], horses [43, 45], ram [43], rabbits (unpublished), and men [46]. The conservation of β -NGF in the semen of males whose related females are classic spontaneous ovulators (i.e. cattle, mare, sheep, and women) confers the biological importance of β -NGF and postulates its involvement with andrology and male fertility.

Administration of OIF/NGF induces ovulation in camelids by triggering LH secretion (reviewed in[47]). Ovulation occurs both with the use of seminal plasma from related and unrelated species [42, 45, 48]. In llamas that failed to ovulate after seminal plasma treatment, an increase in LH pulsatility was observed [45]. The effect of OIF/NGF depends on the route of

administration and the amount given[13, 49-51]. While intramuscular and intravenous administrations are most effective for ovulation induction in most if not all llamas, intrauterine deposition of seminal plasma requires a significantly higher dose to achieve a similar response[50]. In an earlier study, intrauterine administration of seminal plasma was only effective in eliciting the ovulatory response when the uterine lining was abraded[13]. Curettage of the uterine lining facilitates absorption of seminal plasma proteins into circulation and is most likely the method that occurs during natural mating. This dependency on dose to trigger LH secretion, ovulation and CL formation has been reported in *in vivo* [49, 50] and *in vitro* [52, 53] studies.

A luteogenic effect of seminal plasma has been observed in a variety of unrelated species. Using the llama *in vivo* bioassay, corpus luteum formation was observed after intramuscular treatment of seminal plasma from bovine bulls [42], bucks [54], stallions and boars [45]. In cattle, a luteotrophic effect of OIF/NGF or bovine seminal plasma has also been characterized[55, 56] and in prepubertal gilts, transcervical treatment of seminal plasma produced heavier corpora luteum and higher concentration of progesterone[57]. In addition, ovarian weight was significantly greater in mice given seminal plasma in comparison to those treated with saline [58].

The following literature review provides general information about the reproductive physiology in camelids in the context of reflex-induced ovulators and our current understanding of the role of OIF/NGF in semen.

1.1 Reproductive physiology in male camelids

The extant members of the *Camelidae* family encompasses both the larger old world (*Camelus bactrianus* (two-humped) and *Camelus dromedarius* (one-humped)) and new world (*Lama glama* (llama), *Lama pacos* (alpaca), *Lama guanicoe* (guanaco) and *Vicugna vicugna* (vicuna)) species[59]. At birth, both male testicles are fully descended and spermatozoa can be observed in the lumen of seminiferous tubules at approximately two years of age [59, 60]. Puberty is associated with an increase testis diameter, circulating testosterone concentration, spermatozoa in the lumen of seminiferous tubules and the obliteration of preputial adhesions to the penis [61]. Camelids have one of the smallest testes:body size ratio among other livestock animals (0.02-0.03% of total body weight [62]) which is believed to contribute to the low sperm concentration found in ejaculates [59, 63]. The duration of spermatogenesis in camelids is approximately 60 days and is continuous throughout the year [64]. The seminiferous epithelium can be divided into eight distinct stages with progenitor spermatogonia and Sertoli cells in contact with the basal lamina while more mature cells are found towards the lumen [64, 65]. Like other domestic animals, spermatogenesis is influenced by stress induced by poor nutrition, physical injury, and environmental changes [66]. High environmental temperature was associated with seminiferous tubule malformation and a reduction in sperm concentration [67]. Similar to other livestock animals, the epididymis is the sperm reservoir. In camels, sperm were most abundant in the *corpus* of the epididymis (3×10^9), followed by the *cauda* (2.2×10^9), and was less abundant in the *caput*[68]. The accessory genital organs in camelids include paired ampullae, dorsal *corpus* and *disseminate* portions of the prostate, and paired bulbourethral glands [69]. The size and secretory activity of these glands were shown to increase with age [70].

Llamas and alpacas can breed year round but in the Andes, semen characteristics vary with season. While no difference was observed in semen volume, sperm motility or sperm

viability, ejaculates obtained in winter had a greater sperm concentration and less sperm tail abnormalities than those collected in summer [71]. In South America, the breeding season corresponds with forage-growing weather to optimize breeding management [72] and typically occurs between December and March, but has been reported as early as November and as late as May [63, 72-75]. Similarly in China, the breeding period begins at the end of December, when daylight periods are longer and the temperature is low, until mid-April [38]. This period was associated with higher plasma testosterone concentration and greater testes diameter in vicuna [76]. In contrast, the summer season was associated with poor libido, lower testosterone concentrations and a reduction in seminiferous tubule diameter and spermatogenesis in camels [77, 78].

Camelids ejaculate continuously throughout copulation and are often referred to as 'dribble' ejaculators [79, 80]. During intromission, the male moves his pelvis forwards and backwards, a process reported to last anywhere from 3 – 65 minutes depending on the species of camelid [73, 81, 82]. Urethral pulses monitored by digital palpation [80] or transrectal ultrasonography [79] were found to occur throughout the copulation period.

Semen collection in camelids has been attempted by electroejaculation [71], artificial vagina [83, 84], intravaginal condom [62], intravaginal sponge [85], and vaginal aspiration post-mating [62]. Of all these methods, electroejaculation and artificial vagina are most frequently used. Irrespective of the method, great variation has been observed in ejaculates collected within and among males [71, 83, 86].

In addition to low sperm motility and concentration, camelid ejaculates are highly viscous. Mucin 5B appears to be the principle factor responsible for viscosity in camelids as a correlation between its concentration and semen viscosity was found in alpacas [87]. Semen

viscosity can liquefy on its own or with the use of hydrolytic enzymes such collagenase, fibrinolysin, hyaluronidase and trypsin [88, 89]. Of all enzymes, collagenase did not impair sperm motility or viability [89].

1.2 Ejaculation as a nervous reflex

Ejaculation is a spinal reflex that requires the cooperation of sympathetic, parasympathetic and somatic innervation. Stimulation of the hypogastric and splanchnic nerves is required for seminal emission and closure of the internal urethral orifice [90]. Innervation of male reproductive tissue originate from the pelvic, sympathetic chain, mesenteric and dorsal root ganglia [91-94]. Ejaculation is defined as the expulsion of seminal fluids through the urethral meatus and can be divided into two distinct processes: emission and expulsion [95]. Emission incorporates smooth muscle contractions to simulate the secretion of seminal fluids from glandular epitheliae into the pelvic urethra [95]. The coordinated process of emission is dependent on the closure of the neck of the bladder, contraction of seminal vesicles, prostate and bulbourethral gland, followed by contraction of the ductus deferens and epididymides. After emission, the seminal fluid in the proximal urethra is expelled out of the urethral meatus by the process of ejaculation. Expulsion of semen uses sympathetic and somatic nervous stimulation for rhythmic contractions of striated perineal muscles, primarily the bulbospongiosus muscle [95].

The importance of an intact nervous system on male reproductive potential is best illustrated in men with spinal cord injuries. A dysfunction in the ejaculatory mechanism and poor semen quality result from spinal lesions and the severity is dependent on the location of the injury and the degree of damage [96]. The lumbosacral region of the spinal cord is the control center for ejaculation. This is best illustrated by the ability of vibratory stimulation to induce

ejaculation despite spinal cord lesions[97]. The ejaculates from men with a spinal cord injury are inferior in comparison to those collected from normal men in terms of semen volume, sperm motility[96, 98] and morphology [98] while sperm concentration has been reported as normal [98-100] or low [96]. Some of these aberrations in sperm parameters may be the consequence of factors in seminal plasma. The supplementation of seminal plasma from injured men to sperm taken from normal men impaired sperm motility within five minutes of addition [101]. Correspondingly, adding normal seminal plasma to sperm collected from injured men rescued sperm motility within five minutes [101]. Furthermore, sperm collected from injured men at the level of the ductus deferens had a higher motility and viability in comparison to ejaculated sperm while no difference was reported in normal males between ejaculated and aspirated sperm from the ductus deferens[102].

1.3 Seminal plasma and fertility

The male accessory genital glands vary considerably in terms of topographical location, size, morphology and secretions which collectively reflect the diversity of their contribution to male reproduction and the species-specific requirements for fertility. The importance of male accessory genital glands is highlighted whenever there is a loss of their functionality either by surgical removal [103-105] or infection [106, 107]. In rats, pregnancy rates were lowered when the seminal vesicles, dorsal and ventral prostates were removed[108]. Ablation of the coagulating gland had no effect on pregnancy rates and litter size decreased only with seminal vesicle removal[108]. In another study, the ablation of the ventral prostate had no effect on fecundity but the removal of the dorsolateral lobes of the prostate or seminal vesicles induced complete infertility[103]. The removal of seminal vesicles in the house mouse caused a reduction

in pregnancy rate and birthing interval, while the average litter size was not changed; sperm collected in the uterus had lower progressive motility than sham operated males [109].

In the golden hamster, the removal of accessory glands had no effect on pregnancy rates, but delayed embryo cleavage and impaired implantation approximately four days post coitus [104]. In addition, pups born from breedings with males without accessory genital glands displayed abnormal traits such as abnormal physical growth, accelerated acoustic startle, more frequent rearing and premature cessation of nest patch odor preferences[110]. The removal of the ventral prostate reduced pregnancy rates but had no influence on litter size in the golden hamster; in litters born from seminal vesicle ablation, more males were born in the litter[105].

The effect of seminal plasma on sperm motility is controversial. Lower seminal plasma concentration was associated with better sperm motility in stallions with cooling or after cryopreservation [111, 112]. Removal of seminal plasma by centrifugation before long-term cooling improved sperm motility in the ejaculates from stallions that had poor tolerance to cooling and storage; the removal of seminal plasma by centrifugation in ejaculates with a superior tolerance to cooling did not improve sperm motility [113]. In contrast, others report that the presence of seminal plasma during cooled storage did not impair sperm motility but it did cause increased DNA damage [114]. In addition, the removal of seminal plasma had no effect on sperm motility after cooled storage and that total sperm motility was reduced after storage in the absence of seminal plasma [115]. The removal of seminal plasma was beneficial to maintain sperm plasma membrane integrity on fresh and cooled semen which was evident by the hypo-osmotic swelling test [115]. Seminal plasma collected from males of the same species is not the same. The addition of seminal plasma from stallions with high post-thaw viability to spermatozoa before freezing significantly increased sperm motility[116]. The addition of seminal

plasma from stallions with low post-thaw sperm motility to spermatozoa reduced sperm motility in relation to extender-only controls [116]. Seminal plasma from bull cattle with above average fertility improved fertilization capability using epididymal sperm, while no benefit was observed when seminal plasma from bulls of lower fertility was used [117].

The role of seminal plasma proteins on male fertility has been investigated in a variety of species. The comparison of seminal plasma protein profiles in Holstein bulls that were categorized in terms of fertility showed two proteins were present at a higher concentration in more fertile bulls [118]. These two proteins were later identified as lipocalin-type prostaglandin D [119] and the phosphoprotein, osteopontin [120]. Osteopontin secretion was localized in the lumen and epithelial cells of the seminal vesicle and ampulla [121]. Osteopontin is conserved in the seminal plasma of other domestic animals. While osteopontin concentration was positively correlated with fertility in stallions [122], a negative correlation was observed in boars [123]. Osteopontin appears to regulate sperm binding and zona penetration and prevents early capacitation of epididymal sperm. In boars, the addition of increasing doses of osteopontin reduced polyspermy rates in *in vitro* trials but had no effect on sperm motility or viability [124].

In addition to osteopontin, other proteins have been identified as potential biomarkers to predict fertility in stallions. Total protein content in the ejaculate was negatively related to first cycle conception rate [125]. Enzymes found in the ejaculate including malate dehydrogenase, fumarate hydratase, α -enolase, dihydrolipoamide dehydrogenase and citrate synthase were all positively related to first cycle conception rate [125]. The secretion patterns of six seminal plasma proteins change with fertility. Seminal plasma that contained less IGF1 content was associated with the most fertile stallions. Four different isoforms of PLUNC (palate, lung, and nasal epithelium) -associated proteins were negatively related to IGF1 concentrations and had a

positive relationship with first cycle conception rate in seminal plasma[125]. Clusterin was associated with IGF1 concentrations and was negatively associated with fertility. Kallikrein-1E2 was also present in two different isoforms each of which had a negative and positive relationship with overall pregnancy rates [125]. Seminal plasma protein (SP) 1 and 2 were both negatively associated with first cycle conception rates. A cysteine-rich secretory protein 3 (CRISP3) was the only seminal plasma protein to have a positive association with first cycle conception rate[125].

1.4. Reproductive physiology in camelids and other reflex ovulators

All camelids are induced ovulators and as such, do not have an estrous cycle. Consequently, this makes it difficult to determine sexual maturity. In alpacas, ovarian function was detected as early as 10 months of age [126] and can be bred at 12-14 months of age [72]. Body weight has also been used as an indicator of puberty: llamas and alpacas weighing 50-60% their final body weight demonstrate signs of sexual maturity [127, 128]. In spite of these observations, in Peru, llamas and alpacas are not bred until they reach 2 to 3 years of age [59, 128].

1.4.1 Follicular phase

Camelid ovaries are in a constant state of follicular proliferation and regression [74, 129-131]. Ovarian follicles develop in a wave-like pattern throughout the reproductive life of camelids [130, 131]. Ultrasonography and histology sections have been used to demonstrate that this pattern of follicular development occurs in spontaneous and induced ovulators [130-139]. In cattle and camelids, FSH induces follicle recruitment [140, 141], [142]. There is a period of

overlap between successive waves but no alternating pattern exists in waves emerging from either ovary [130, 143, 144]. Each wave can be divided into a growing, static and regressing phase [133]. Follicular waves in New world camelids are comparable in duration. The growth phase spans approximately 7-8 days in llamas [130, 145], alpacas [144] and guanaco [146]. No change in the diameter of the dominant follicle was observed for approximately 5-7 days before regressing [130, 131, 146, 147]. The lifespan of anovulatory follicular waves ranges from 20-25 days in llamas and with a mean inter-wave interval of 19.8 ± 0.7 days [130]. Shorter follicular waves may be the consequence of high levels of progesterone in circulation (pregnant or nonpregnant luteal phase), lactation, or malnutrition [130, 148]. The reported length of a follicular wave is longer, however, in Old world camels [149] and shorter in vicuna [139].

Administration of LH (to induce ovulation) or the use of ultrasound-guided follicle aspiration of all follicles with a diameter of ≥ 5 mm, are methods used to synchronize wave emergence within 2 days treatment [150]. Follicles ≥ 6 mm are LH dependent and capable of ovulation [151, 152] and selection of the dominant follicle represses further growth of subordinate follicles [130, 144, 153]. During the follicular phase, circulating levels of progesterone are low because of the absence of a functioning CL. Circulating levels of estradiol $17-\beta$ are associated with follicular growth and peak concentrations were found prior to when the dominant follicle reaches its maximum diameter [146, 149, 154].

Follicle size does not influence sexual receptivity; females with only 3 mm follicles were equally receptive to male advances as those with follicle diameters ≥ 7 mm [155]. Females that are receptive take the sternal recumbent position on their own or after the male exerts force on their hindquarters [81]. Once the female is in the recumbent position the male enters a half-sitting mounting position at her rear and adjusts his pelvis to facilitate intromission.

1.4.2 Ovulation

Ovulation is the physiological process whereby rupture of an ovarian follicle results in the release of an oocyte that may be used for subsequent fertilization by spermatozoa. This event is associated with differentiation of follicular cells to luteal cells and rapid changes in gene expression, angiogenesis, hormone secretion and cell morphology [156-158]. Ovulation in camelids is preceded by a rise in circulating levels of LH secreted by gonadotrope cells within the adenohypophysis. Ovulation-induction after sterile or non-sterile mating, GnRH, OIF/NGF, seminal plasma or exogenous LH occurs approximately 30 hours after treatment [159, 160]. The effect of LH is mediated by binding to its receptor on granulosa and theca cells, which initiates signaling pathways involved in ovulation and luteinization [161-163]. The LH surge regulates gene expression by turning off genes involved in folliculogenesis and initiating the expression of other genes, such as the early growth response protein -1 (EGR-1) [164]. The early growth regulatory factor-1 (EGR) is a transcription factor in granulosa cells and mice expressing the null allele for EGR-1 were infertile due to impaired development of the anterior pituitary gland [164, 165]. Prostaglandin and enzyme synthesis involved in follicle rupture are also LH dependent [164]. The LH surge also induces expression of the proteases ADAMTS-1 (A Disintegrin And Metalloproteinases with Thrombospondin motifs 1) and cathepsin-1 in granulosa cells which are believed to be involved in the degradation of the follicular wall [166]. The LH surge induces the expression of the progesterone receptor. Blocking the action of the progesterone receptor impairs ovulation in rats [167].

Following an LH surge, the LH β secretory granules are depleted from gonadotrope cells and replenishment of these granules is only partial after 48 hours [168]. To support this notion, a

second LH-secreting stimulus given six or twenty-four hours after GnRH or copulation failed induce LH release in llamas and alpacas [160].

Two main hormones are used to trigger LH secretion and ovulation induction in camelids: GnRH and OIF/NGF. Gonadotropin-releasing hormone (GnRH) is viewed as the central regulator of LH pulses in mammals [169]. The distribution of neurons that secrete GnRH varies among species but they are commonly concentrated in the preoptic and medial basal region of the hypothalamus [31]. GnRH is released into primary portal capillaries in the median eminence and is targeted to gonadotrope cells via the hypothalamo-hypophyseal portal system [170]. Exogenous GnRH is an effective hormone for ovulation induction and its function appears necessary to elicit this effect in camelids [48, 150, 171, 172]. Llamas that were given a GnRH antagonist prior to OIF/NGF administration, showed no increase in circulating LH levels and failed to ovulate[171]. The impediment of GnRH was evident by annihilating LH release [171]. The secretion of GnRH in relation to mating stimuli has also been shown in other induced ovulations. Push-pull perfusion sampling of the medial basal and anterior region of the hypothalamus showed an increase in GnRH concentration from 1.15 ± 0.29 pg/mL to 106.67 ± 37.42 pg/mL in rabbits before and after coitus, respectively [173]. A 3-fold reduction in residual GnRH in hypothalamic neurons was observed in voles after mating [174] and in ferrets, mechanical stimulation of the vagina and cervix caused abrupt release of GnRH which was evident by a 50% depletion of GnRH neurons prior to the LH surge [16].

Most of the information regarding the influence of nerve growth factor (NGF) on LH secretion in camelids has either been reported as ovulation-inducing factor (OIF) or within the context of seminal plasma. This neurotrophin is found in camelid semen and is the most abundant protein in llamas and alpacas semen [43]. The administration of seminal plasma or

OIF/NGF purified from seminal plasma, initiates the ovulatory cascade by stimulating LH secretion [48, 49, 171]. The response to this hormone is rapid and a significant increase in circulating LH levels has been reported as early as fifteen minutes after treatment [48, 175]. A similar pre-ovulatory LH secretion pattern was observed in llamas that were administered rabbit or llama seminal plasma [54]. In females that failed to ovulate, there was an increase in LH pulsatility with the administration of either horse or pig seminal plasma [45].

In reflex ovulators, there has been little evidence that estradiol production contributes to the preovulatory LH surge. In rabbits, estradiol-17 β concentration remained unchanged during periods of estrous and pseudopregnancy [176] and the administration of exogenous estradiol had no effect on LH secretion in rabbits as well as ferrets [177, 178]. In one study, however, the possible role for estradiol was reported to augment the sensitivity of LH to GnRH [179]. Luteinizing hormone levels following OIF/NGF treatment was lower in ovariectomized llamas than in intact llamas. Estrogen priming did improve the LH response but was still significantly lower than what was observed in intact animals [180]. A clear relationship between estradiol concentration and follicle size is evident [138, 139, 181] and thus, at the time of coitus, a dominant follicle and elevated levels of estradiol are present. Estradiol levels decline and remain low 48h after ovulation [181]. A similar relationship was evident in cats, where levels of estradiol-17 β fluctuated during estrous with the growth of the follicle, but appeared to have no effect on the magnitude or duration of the LH surge [182].

1.4.3 Luteogenesis

In response to high levels of LH production and ovulation, luteinization of theca and granulosa cells from the ovulated follicle forms the corpora luteum [183]. During the luteal phase, progesterone concentrations are high (> 1 ng/mL) which is evident by unreceptive females and regular emergence of short-spanning, anovulatory follicular waves [130]. Corresponding with CL formation and growth, significantly higher levels of progesterone are observed two days after ovulation (D4 after treatment) and continues to increase until the CL reaches its maximum diameter by Days 8-9 after treatment [184-186]. There is a strong correlation between the diameter of the preovulatory follicle and subsequent diameter of the corpus luteum [187].

1.4.4 Placentation and Luteolysis

If mating successfully results in pregnancy, the CL is maintained throughout the entire gestation length [187] which spans 331-347 days in llamas [63] and 325-361 days in alpacas [73]. The placenta in camelids is described as epitheliochorial and diffuse, which is different from other ruminants but similar to what is found in pigs [188, 189]. Maternal recognition of pregnancy occurs between Days 8-10 in camelids [190]. Contrary to cattle, where interferon tau is the maternal signal for pregnancy [191-193], estradiol production by the blastocyst signals pregnancy in camelids [194]. The production of estradiol occurs as early as Day 7 in llama blastocysts, increases 4-fold by Day 11 and a dramatic 200-fold increase is observed by Day 13 [194]. This period is associated with a slight decrease in progesterone concentration in circulation [190]. Correspondingly, estrogen receptors (α and β) have been localized to the CL and uterus of llamas, which further supports the role of estrogen in pregnancy signalling [195]. Exogenous administration of estradiol on Days 8 and 9 (D0= GnRH treatment), however, did not improve

pregnancy rates when used in an embryo transfer program [196]. Estrogen production precedes blastocyst elongation and stimulates calcium and prostaglandin secretion into the uterine lumen [197]. In llamas, blastocyst elongation begins around Day 11 and is completely filamentous by Day 13 [194].

Prostaglandin $F_{2\alpha}$ is the physiological luteolysin in camelids when pregnancy does not occur. The pulsatile release of prostaglandin ($PGF_{2\alpha}$) from the uterus occurs as early as Day 7 and peaks between Days 8-11 in llamas [198, 199]. Correspondingly, the first significant decrease in CL diameter and progesterone concentration in nonpregnant llamas occur on day 9 and 10, respectively [187]. Prostaglandin $F_{2\alpha}$ secretion is by the uterus. Prevention of luteolysis and thus, maintenance of the CL lifespan can occur by the removal of either the right or left uterine horn. The left uterine horn can cause luteolysis in both ovaries while right uterine horn can induce luteolysis only in the ipsilateral ovary [200, 201]. These results demonstrated a local and systemic luteogenic pathway in camelids. This different activity between uterine horns is the consequence of the angioanatomy; a branch of the right uterine artery supplies the left horn and a corresponding major vein that originates from the left horn, terminates as a branch of the right uterine vein [201]. Prostaglandin secretion by the uterus and its effect on the CL is mediated by the vascular countercurrent diffusion mechanism whereby the proximity of the ovarian artery and veins allows rapid exchange of metabolites into circulation [201]. The response to $PGF_{2\alpha}$ is dependent on the age of the CL. Luteolysis was not observed in llamas given prostaglandin 3 or 4 days after GnRH administration and less than 20% showed complete luteolysis on Day 5 [202]. Complete luteolysis, evident by the reduction in progesterone concentration and CL diameter (via ultrasonography), was observed in all animals when prostaglandin was administered six days after GnRH treatment [202]. Although the exact mechanism for prostaglandin secretion is

unclear, oxytocin, which is secreted by the pituitary or luteal cells, was shown to stimulate prostaglandin pulsatility [203, 204].

1.5 Nerve growth factor and reproductive cycle

The importance of NGF and its receptors is apparent by the degree of protein conservation among species including primates [205, 206], bovids [44, 207, 208], rodents [209-212], camelids [39, 40], xenopus [205], serpents [205, 213], fish [205] and aves [205]. The biological roles that NGF plays within the female reproductive system are crucial for follicular development, ovulation induction and uterine changes throughout the estrous cycle.

The nervous tissue density of the uterus changes throughout the estrous cycle and in many species, pregnancy induces denervation of the uterus [214]. In hamsters, NGF detection increased from low on Day 2 to very high on Day 4 in luminal epithelial and stromal cells, while no change was detected in the glandular epithelial cells [214]. High NGF levels (Day 4) corresponded with high estradiol-17 β levels and the administration of exogenous estradiol-17 β further increased the immuno-staining intensity of NGF in epithelial and stromal cells in the uteri of ovariectomized hamsters [214]. The expression pattern of trkA (its high affinity receptor) appears to follow NGF more than p75 (its low affinity receptor) in stromal cells: high NGF and estradiol-17 β levels corresponded with very strong trkA detection in stromal cells but had no effect on p75 [214]. The administration of estradiol-17 β to ovariectomized hamsters increased the detection of both receptors in the endometrium and p75 in the glandular epithelium [214]. In rats, the expression of trkA was inversely related with mean estradiol concentrations[215]. High expression of trkA occurred during the morning of diestrus when mean serum levels of estradiol

was low and low expression was detected during the afternoon of proestrus when mean concentrations of estradiol was at its highest [215]. In this study, no changes in NGF expression was reported [215].

Changes in circulating estrogen concentrations have been shown to influence cholinergic neurons of the basal forebrain in rats and estrogen replacement can improve basal forebrain cholinergic function [216-219]. Colocalization of steroid and the p75 common neurotrophin receptor in cholinergic nuclei demonstrates a relationship between estrogen and NGF on nervous tissue function [220]. A disruption of the mouse NGF gene caused severe cell loss in both sensory and sympathetic ganglia, but the continued differentiation of basal forebrain cholinergic neurons demonstrated that other neurotrophins, such as BDNF [221-223], can rescue nervous tissue function in the absence of NGF [224]. Lower estrogen levels associated with aging and ovariectomy resulted in decreased trkA expression in medial septum neurons which may impair the responsiveness of the cholinergic neurons to NGF [225]. In addition to the brain, rats given estradiol valerate formed polycystic ovaries which were attributed to an increase of sympathetic ovarian neurons, higher NGF and p75 levels in the ovaries and adrenal glands 30 days post injection [226-229]. Normal estrous cyclicity and ovulation in rats with polycystic ovaries was restored using NGF antiserum or by transection of the superior ovarian nerve [226, 227]. Impaired follicular development, loss of sympathetic innervation, decrease in androgen and estradiol production, delayed puberty and disrupted estrous cycle were also observed in neonatal rats immunized against NGF[230].

The organization and development of the ovarian follicles is NGF dependent. Histological assessment of homozygous NGF-mutant mice showed improper organization of ovarian follicles, increased number of naked oocytes, and a significant reduction in the number

of primary and secondary follicles, with no change in the number of primordial follicles [231]. These histopathological changes in morphology were not caused by or had an influence on the adrenal or pituitary gland[231]. Both receptors for NGF (trkA and p75) have been localized to rat ovaries and their expression levels change in relation to ovarian follicular dynamics [231, 232]. In humans, NGF treatment was associated with delayed luteogenesis. Human granulosa cells respond to NGF with increased estradiol secretion and expression of FSH receptor mRNA, and reduced progesterone output within 18 h of treatment [233]. A luteotrophic effect of NGF was shown *in vitro* when bovine lutein cells stimulated progesterone secretion with NGF treatment [234].

2.0 HYPOTHESES AND OBJECTIVES

2.1 General Hypothesis

The overarching hypothesis of the work described in this thesis is *the concentration or total abundance of OIF/NGF in semen is related to male fertility in South American Camelids*.

2.2 General objective

The main goal of the work described in this thesis is *to elucidate whether OIF/NGF concentration or total abundance in semen can be used to predict male fertility in South American Camelids*.

2.3 Specific objectives

Based on the following observations:

- Seminal plasma proteins have been associated with male fertility and sperm parameters in cattle[118, 120, 235, 236], horses[122, 125] and pigs [123] ;
- Male accessory genital gland secretions and relationship with fecundity are variable among mammals [103-106, 108, 109, 237];
- Administration of seminal plasma, purified OIF/NGF from seminal plasma and NGF from mouse salivary gland induces ovulation in female llamas and alpacas [39, 40, 45, 54, 238];
- The ovulation-induction effect of seminal plasma or OIF/NGF is dose dependent in *in vivo* and *in vitro* bioassays [49, 50, 52, 239];

- OIF/NGF promotes a longer lasting LH surge in comparison to that characterized following GnRH treatment in llamas and alpacas [175, 238, 240];
- OIF/NGF is luteotrophic evidenced by producing larger CL diameter and more progesterone [175, 238, 240];
- OIF/NGF exerts a luteotrophic effect by altering LH secretion and enhancing CL vascularization [175].

The primary objectives of the first study (Chapter 3) was to:

- Characterize the source of OIF/NGF secretion in male accessory genital glands;
- Develop a method for measuring OIF/NGF concentration in semen.

The primary objective of the second study (Chapter 4) was to:

- Elucidate the breeding and semen parameters which were related to OIF/NGF concentration and total abundance in alpaca semen.

The primary objective of the third study (Chapter 5) was to:

- Determine if a higher OIF/NGF concentration in semen was associated with high ovulation, pregnancy and live birth rates.

The primary objective of the fourth study (Chapter 6) was to:

- Determine if higher OIF/NGF abundance in an ejaculate was associated with more luteogenic CL;
- To elucidate the efficiency of using Power Doppler Ultrasonography to assess CL form and function between pregnant and nonpregnant animals.

3.0 SOURCE AND ABUNDANCE OF OVULATION-INDUCING FACTOR / NERVE GROWTH FACTOR (OIF/NGF) IN THE SEMINAL PLASMA AMONG MAMMALIAN SPECIES

3.1 Abstract

The seminal plasma protein, ovulation-inducing factor (OIF), is the well-characterized neurotrophin, nerve growth factor (NGF). The administration of exogenous OIF/NGF or seminal plasma in camelids initiates the ovulatory cascade through the release of luteinizing hormone. An attenuated ovulatory response has also been documented in camelids using seminal plasma from cattle, horses and pigs. Thus, we hypothesize that this protein is a widely conserved component of semen among mammals but is most abundant in camelid ejaculates. The objectives of the study were to compare the presence and distribution of OIF/NGF in male reproductive organs and to determine the abundance of OIF/NGF in the ejaculates of species representative of both spontaneous and induced ovulators. Immuno-histochemical analysis was performed on tissue from llamas, rats, cattle, bison, elk, and white-tailed deer, and the abundance of OIF/NGF in the ejaculates of camelids (llamas and alpacas), cattle, horses and pigs were quantified by radioimmunoassay. The density of immuno-reactive material among accessory genital glands was most abundant in the following regions: llama prostate gland (epithelium and gland lumen); rat coagulating gland (anterior prostate; blood vessel lumen); bovine ampulla and vesicular glands (epithelium and lumen); bison and elk ampulla (epithelium); and white-tailed deer (body of the prostate). Of all tissues, the llama prostate gland contained the most intense and abundant immuno-reaction. Quantitative analysis showed that the concentration of OIF/NGF in seminal plasma in camelids was higher than that of bulls ($P<0.05$). In llamas and alpacas, OIF/NGF

represented 27% of total seminal proteins and less than 1% in bulls ($P < 0.05$). Reduced OIF/NGF levels in bull semen corresponds with previous reports where a lower ovulatory response in camelids was detected. These observations further support that OIF/NGF behaves in a dose dependent manner and that OIF/NGF may have greater importance, in terms of reproductive physiology, in induced than spontaneous ovulators. Although OIF/NGF was detected within the lumen or glandular epithelium of at least one accessory gland of all species investigated, we were unable to detect OIF/NGF in the ejaculates of either horses or pigs. Thus our general hypothesis that OIF/NGF is conserved among all mammals was only partially supported.

3.2 Introduction

Semen is comprised of spermatozoa carried in fluid derived from accessory genital glands and, to a lesser extent, the epididymis and testis. The accessory genital glands are composed of paired ampullae of the ductus deferens, paired vesicular glands, the body and disseminate parts of the prostate, and paired bulbourethral glands [241]. These exocrine glands vary considerably among species in terms of size, secretions, and anatomical and histological structure. For example, while all glands are present in cattle [242] and horses [243], the vesicular glands are absent in camelids [81], dogs, and cats [241]. The prostate, whether it is confined in a solid organ (body) or dispersed in the mucosa around the pelvic urethra (disseminate), is the only accessory genital gland that is present in all mammals [241]. In light of the diversity in structure, the contribution of each gland and the degree to which its secretions influence male fertility remains to be elucidated.

Nerve growth factor (NGF) is a 26 kDa homodimer protein that is classically known for its involvement in neuronal survival and maintenance [35]. However, this protein has been implicated in several biological pathways outside of nervous system. Its detection in semen has led to a new line of research that focuses on its effects on the female reproductive endocrine system [39, 44, 208, 211, 244, 245]. Referred to originally as ovulation-inducing factor (OIF) in seminal plasma for its potent ovulation-eliciting effect in camelids [48, 246], results of recent studies revealed that it is β nerve growth factor (NGF) [39]. Results from a series of studies have demonstrated that the administration of seminal plasma or purified OIF/NGF from seminal plasma triggers a surge in circulating concentrations of LH which leads to ovulation and corpus luteum (CL) formation (reviewed in [47]). The endocrine response is related to the quantity of OIF/NGF or seminal plasma that is given [49]: *in vitro*, a pituitary culture augmented its LH

release when treated with higher doses of OIF/NGF or seminal plasma [52, 53]. This response was paralleled *in vivo*: the preovulatory LH surge, ovulation rate and diameter of the CL were greater with increasing doses of OIF/NGF [49].

It appears that OIF/NGF is the primary stimulus for ovulation-induction in camelids replacing the dogmatic view that ovulation occurs via penile stimulation of somatosensory neurons in vagina [1]. However, it is evident that OIF/NGF has more than one function in regards to male and female reproduction and these functions vary among species. Nerve growth factor is involved in sperm development and maturation. Nerve growth factor and its receptors TrkA and p75 were found in developing sperm cells in the testis of mouse and rat [247], golden hamster[248], cattle [249], alpaca [250], Japanese monkey [251], and human [252, 253]. Of all of these species, only one (alpaca) is classified as an induced ovulator whereby OIF/NGF it been shown to cause ovulation in their respective females [47, 254]. While the direct effect of OIF/NGF on camelid sperm has not been reported, incubation of NGF with sperm cells *in vitro* increased sperm motility in a dose dependent manner and increased the number of cells that underwent the acrosome reaction following capacitation in the golden hamster[248]. In humans, NGF promoted sperm motility [252, 255], however, the quantity of NGF was not different comparing ejaculates that had normal and low sperm motility [46]. Exogenous NGF had no effect on mitochondrial activity or acrosome reaction, in cattle, but did improve sperm vitality[249]. Thus, it may be likely that the function of OIF/NGF in males of spontaneous ovulators is to assist sperm while in the female tract, and not necessarily induce changes in female physiology.

Historically, seminal fluids and its constituents have been deemed essential for sperm transport, nutrition, capacitation and fertilization (reviewed in [256, 257]). The necessity for

accessory genital glands is evident by the consequence of their removal in an assortment of species. Mating of intact female mosquitoes with males whose accessory glands were removed caused a reduction in the total number of fertile eggs laid [237]. The removal of vesicular glands in mice had a negative effect on sperm progressive motility and resulted in lower pregnancy rates [258]. In a later mouse study, the removal of vesicular and prostate glands diminished pregnancy rates, while no effect was observed when the coagulating gland was ablated [259]. Excision of the bulbourethral glands in llamas impaired sperm binding to the oviduct and it was proposed that proteins secreted from this gland were needed for the formation of the oviductal-sperm reservoir [260]. These observations suggest that the importance of respective accessory genital glands is species-specific and the product of one gland may be more expendable than another in terms of male fertility. The comparative distribution, relative abundance, and importance of OIF/NGF in the male reproductive tissues have not been examined critically.

Several assays have been used to detect NGF within tissue and to measure NGF in fluid secretions. These include the differentiation of immortalized rat pheochromocytoma cells (PC₁₂) [39, 261], chicken dorsal root ganglion [262], enzyme-linked immunosorbant assay [46, 263], radioimmunoassay [262] and llama bioassay [45, 48, 49, 54]. Qualitative methods, such as immunohistochemistry provide important information of detection and distribution, but signal intensity can be skewed by tissue preservation, antibody affinity, staining procedures and experimenter bias. Quantitative assays, such as radioimmunoassay where known amounts of labeled ligand competes with unlabeled ligand for antibody binding sites within the sample of interest, provide repeatable numerical assessment of the amount of the antigen of interest [264].

Abundant levels of NGF have been detected in the salivary gland of the adult male mouse [36, 210], snake venom [265, 266] and the prostate gland of guinea pigs [211]. The concentration

of NGF can be influenced by age, gender, and endocrine status. The synthesis of NGF in salivary glands begins after puberty and is higher in males than in female mice [35, 37]. Elevated levels of NGF in circulation appear to be related to the release of NGF from salivary glands in response to psychosocial behaviour and stress [209, 263, 267, 268].

Based on the overwhelming evidence in literature, we hypothesize that OIF/NGF is a widely conserved component of semen but it is most abundant in camelid ejaculates. Thus, the objectives of the present study were to determine the source of OIF/NGF secretion among different mammalian species and to quantify its abundance in the ejaculate using qualitative (immunohistochemistry and PC12 differentiation) and quantitative (radioimmunoassay) methods. To our knowledge, OIF/NGF storage and secretion sites that contribute to the mammalian ejaculate have not been reported in llamas.

3.3 Materials and Methods

Experimental procedures were performed in accordance with the animal care protocols established by the University of Saskatchewan.

3.3.1 Tissue sources of OIF/NGF in the male reproductive organs

The male reproductive organs from adult male llamas, rats, bulls, bison, elk and white-tailed deer were used (n=2 males/species). Tissues were dissected within one hour of euthanasia and were fixed overnight at 4°C in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M monophosphate buffer (pH 7.4). Tissues were processed to obtain 5 µm-thick paraffin sections on poly-L-lysine coated glass slides.

3.3.2 Immunohistochemistry

Slides were washed three times each, first in xylene and then in absolute ethanol. Slides were dipped in a successive gradient of ethanol solutions (95%, 70% and 50%), rinsed with distilled water (five minutes in running water) and then immersed in phosphate buffered saline (PBS; 15 minutes). Slides were then incubated with pepsin (2 mg/mL in 0.01N HCl; Sigma-Aldrich, Oakville, Ontario, Canada) inside a humidified chamber (for antigen retrieval), rinsed with distilled water (as described above) and immersed in hydrogen peroxide (10% in methanol; for endogenous peroxidase blocking) for 45 minutes. Slides were again rinsed with distilled water for one hour at room temperature before blocking with 5% BSA (Sigma-Aldrich, Oakville, Ontario, Canada). The primary antibody for immuno-histochemical localization of NGF was polyclonal rabbit antiserum raised against human β -NGF (1:400 in 1% BSA for all tissues except for rat (1:200); Santa-Cruz Biotechnology Inc., Dallas, Texas, USA). Slides were incubated overnight at 4°C then washed three times for five minutes each with phosphate buffered saline and incubated inside a humidified chamber with the secondary antibody 1:400 in 1% BSA (polyclonal goat anti-rabbit IgG-HRP; Daka Canada, Inc. Burlington, Ontario, Canada). VECTOR VIP peroxidase substrate kit (Vector Laboratories, Inc; Burlington, Ontario, Canada) was used for colour development and VECTOR Methyl green (Vector Laboratories Inc.) was used as a counter stain. VectaMount permanent mounting medium was used for slide preservation (Vector Laboratories Inc). Slides incubated with 1% BSA without the primary antibody were used as negative controls, and slides of adult rat dorsal root ganglia or llama prostate gland were used as positive controls. Adjacent sections were stained with hemotoxylin and eosin for normal histology assessment.

The overall presence and distribution of positively labeled tissue were examined by light microscopy at low magnification (400x); high magnification was used for analysis of intensity and cellular localization (1000x). A minimum of 15 fields were visualised for each slide for the assessment of positive cells in the mucosa, submucosa, muscularis and serosa/adventitia layers. All tissue sections were analyzed by the same observer and scored based on NGF-staining intensity as +++ (very strong), ++ (strong), + (moderate to weak) ± (faint), - (absent), or ND (not determined).

3.3.3 .Semen collection and handling

Ejaculates (n=69) were collected from male llamas (n=5) and alpacas (n=12) using an artificial vagina and phantom mount [84] during June and July (summer) near Saskatoon, Saskatchewan (52°N, 106°W, and 482 meters above sea level). Briefly, males were introduced to a receptive female and were allowed to mount. Once libido was stimulated, the male was transferred to an adjacent phantom mount fitted with an artificial vagina. Males included in the study were selected from larger groups at each of 3 farms on the basis of acceptance of the phantom mount and artificial vagina. Successive ejaculates from individual males were collected with at least one full day of rest between collections. Ejaculates were centrifuged at 400 x g for 15 minutes and the supernatant was centrifuged again for 1500 x g for 15 minutes. The seminal plasma was decanted, and an aliquot was examined under a light microscope. If spermatozoa were detected, the centrifugation process was repeated until no spermatozoa were detected in the seminal plasma [48, 269]. The seminal plasma of individual ejaculates was then stored at -80°C.

Semen was collected from bovine bulls by electroejaculation (n=53 ejaculates; n=36 bulls), from stallions by artificial vagina (n=6 stallions, n=4 ejaculates/ stallion), and from boars

by the gloved-hand method (n=4; n=4 ejaculates/boar), as previously described [45]. The gel fraction of equine and porcine ejaculates was removed immediately after collection. Ejaculates were centrifuged and seminal plasma was decanted and examined to ensure the removal of spermatozoa, as described for llamas and alpacas. Seminal plasma was stored at -80°C. Upon thawing, an aliquot of seminal plasma was pooled within species for use in radioimmunoassay development and the PC12 bioassay; quantification of OIF/NGF was performed on individual ejaculates.

3.3.4 OIF/NGF purification and in vivo bioassay

After thawing, pooled llama/alpaca seminal plasma was eluted by hydroxyl apatite column chromatography to obtain a partially purified fraction that was furthered purified by FPLC [39, 269]. Approximately 6 mg of purified llama OIF/NGF was diluted in phosphate buffered saline (pH 7.4) to a stock solution of 1.0 mg/mL. The diluted, purified OIF/NGF was stored in 10 µL aliquots at -80°C.

A llama *in vivo* bioassay was used to validate the bioactivity of purified OIF/NGF, as described previously [269]. Ovarian follicular development of mature llamas (n=6) was monitored for at least three consecutive days by transrectal ultrasonography. When a growing follicle ≥ 7 mm in diameter was detected, the llama was assigned randomly to one of two groups and treated intramuscularly with 2 mL of purified OIF/NGF (500 µg in saline) or saline (n=3/group, Day 0 = day of treatment). Llamas were examined by transrectal ultrasonography on Day 3 and Day 7 to detect the disappearance of the ovulatory follicle and the subsequent formation of the corpus luteum.

3.3.5 *In vitro* bioassay

Immortalized rat pheochromocytoma (PC12) cells were cultured according to the method previously described [261]. Briefly, the PC12 cells were grown in a 75 cm² Falcon cell culture flask (BD Bioscience, San Jose, California, USA) at 37°C in 5% CO₂ in RPMI 1640 medium (Sigma-Aldrich, Oakville, Ontario, Canada) with 10% heat-inactivated horse serum (Life Technologies, Burlington, Ontario, Canada), 5% fetal calf serum (HyClone; Thermo Scientific, Logan, UT, USA), 25 U/mL penicillin, and 25 µg/mL streptomycin. Cells were cultured *in vitro* on laminin-coated 6-well tissue culture dishes at a concentration of 2 x 10⁶ cells/ well (BD Bioscience, San Jose, California, USA) for 24 hours before treatment.

To detect the presence or absence of OIF/NGF in the seminal plasma of different species, the culture medium was aspirated and replenished with fresh medium containing 50 ng/mL recombinant NGF (Sigma-Aldrich, Oakville, Ontario, Canada; positive control), 1 µg/mL seminal plasma-derived OIF/NGF, and 5 µL of undiluted seminal plasma from llamas/alpacas, bulls, horses or pigs (3 wells/treatment). As the amount of OIF/NGF was unknown in each sample, a volume of 5 µL was chosen arbitrarily. All media with respective treatments were passed through a 0.22 µm filter (Millipore, Billerica, MA, USA) immediately before use. Two days after the start of *in vitro* culture, the medium was replenished with respective treatments. Cells were observed daily to monitor changes in cell morphology using an inverted microscope at 400x magnification. Four days after the start of *in vitro* culture, the wells were scored as differentiated or undifferentiated. Cells were scored as 'differentiated' if they contained dendritic processes that were $\geq 2 \times$ the length of the cell body.

To confirm that PC12 differentiation was associated with OIF/NGF in seminal plasma, rabbit polyclonal β - NGF antibody (Santa-Cruz Biotechnology Inc.) was added to the culture

medium. Cells were cultured in the similar manner as described above. Following the 24 hour culture period, cells (n=4 wells/group) were given fresh medium containing 1 µg/mL purified OIF/NGF or Cytochrome c. Cytochrome c was chosen as a negative control due to its similar molecular weight and isoelectric point as NGF. At the time of treatment, rabbit polyclonal β -NGF antibody (1:1000; Santa-Cruz Biotechnology Inc.) was added to half of the wells (n=2/group). Two days after culture, the medium was replenished with respective treatments. Cells were monitored (by an inverted microscope) for four days after the first treatment. Cells were scored as 'differentiated' if they contained dendritic processes that were $\geq 2x$ the length of the cell body. The total number of differentiated cells in 10 microscopic fields (at 40x magnification) per well (total of 20 fields per treatment group) were counted.

3.3.6 Radioimmunoassay

Purified, biologically active OIF/NGF isolated from pooled llama/alpaca seminal plasma was labeled with ^{125}I by the chloramine-T method [270]. Phosphate buffer (0.25 M; pH 7.5) was prepared using sodium dihydrogen orthophosphate (monobasic) and disodium hydrogen orthophosphate heptahydrate (VWR, Radnor, Pennsylvania, USA). A total of 2 µg of purified OIF/NGF was added to 20 µL of 0.25 M phosphate buffer. Dissolved protein was then added to ^{125}I (1 mCi; Perkin Elmer, Massachusetts, USA). Chloramine T was added to the reaction, vortexed and incubated at room temperature for 90 seconds. Sodium metabisulfite was added to stop the reaction. The iodinated peptides were purified by column chromatography (PD-10 column; Bio-Rad Laboratories, Inc., Hercules, California, USA). Phosphate buffered saline with gel was used as an eluent and 1 mL fractions were collected into 12 x 75 mm borosilicate tubes. Radioactivity was assessed in 5 µL aliquots of each fraction by gamma-counter.

Serial dilutions of recombinant human NGF β (Sigma-Aldrich, Oakville, Ontario, Canada), purified OIF/NGF, and llama seminal plasma were made and combined with four different concentrations of rabbit polyclonal β -NGF antibody (Santa-Cruz Biotechnology Inc.) to optimize binding specificity. The working dilution of 1:1000 rabbit β -NGF polyclonal antibody was chosen for all subsequent studies. To determine cross-reactivity with other neurotrophins, serial dilutions of purified brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) were made and combined with 1:1,000 rabbit NGF antibody. Specificity was also tested by comparing the specific binding after the addition of either llama OIF/NGF or BSA to different dilutions of llama blood plasma, and llama or bull seminal plasma. Llama blood was collected by jugular venipuncture into heparinized tubes.

Antibody binding was tested by comparing the profile of displaced iodinated OIF/NGF from anti-NGF antibody binding sites using increasing volumes of seminal plasma from llamas/alpacas, bulls, horses and pigs. Seminal plasma dilutions were made with phosphate-buffered saline and 1% polyethelene glycol (PEG) buffer. The amount of OIF/NGF in samples (unknowns), standards, and references (25 and 100 μ g for low and high references, respectively) were measured in duplicate. The standard curve ranged from 0 to 200 μ g/mL. A volume of 0.1 mL of sample was placed in glass tubes and incubated with rabbit NGF antibody (1:1000) overnight at 4°C. The following day, 0.1 mL of 125 I-NGF (containing 10,000 cpm) was added, vortexed, and incubated at 4°C overnight. Goat-anti-rabbit serum (0.5 mL; 1:500) and 5% PEG (0.5 mL) was made in-house [271] and were added to each tube, vortexed, and incubated at 4°C overnight. Tubes were centrifuged at 3000 x g and the supernatant was decanted. The relative amount of unbound tracer in each tube was measured by gamma-counter.

3.3.7 Total protein quantification

Total protein concentrations for llama/alpaca and bovine seminal plasma was evaluated by Bradford's method [272] (Bio-Rad Laboratories, Mississauga, Ontario, Canada). A standard curve was generated using bovine gamma-globulin standard (Bio-Rad Laboratories). Seminal plasma samples were diluted in saline (v:v) 1:2, 1:4, 1:10, 1:25, 1:50 and 1:100 and values which fell within the numerical values of the standard curve were used.

3.3.8 Statistical analyses

Student's t test was used for comparison of OIF/NGF and total protein concentration between llama/alpaca and cattle seminal plasma samples by radioimmunoassay. Equine and porcine samples were excluded due to low to absent detection of OIF/NGF. Analysis of variance (ANOVA) using PROC MIXED procedure was used to compare PC12 differentiation among groups. Tukey's multiple comparison was used as a post-hoc test when treatment means were significant ($P < 0.05$). All statistical analyses were made using SAS software (SAS, Statistical Analysis System Institute Inc., Cary, NC, USA).

3.4 Results

3.4.1 OIF/NGF Immunolocalization- Llama

Immuno-staining of β NGF was detected in the connective tissue of all reproductive organs (Table 3.1). The detection of OIF/NGF was greatest in the body and disseminate parts of the prostate gland (+++). The protein was localized in the apical region of epithelial cells and frequently found within the glandular lumen of the prostate gland (Fig. 3.1). On no occasion was

OIF/NGF detected solely at the basement membrane. Its subnuclear detection was evident only when OIF/NGF was present throughout the cell. Ovulation-inducing factor/ NGF appeared to be stored in secretory vesicles of different sizes. The scarcity of OIF/NGF-negative vesicles suggests that most if not all acini contained OIF/NGF. The presence of immuno-reactive material in the epithelium was omnipresent in the body of the prostate gland. Based on the appearance and size of epithelial cells and their nuclei, all cells appeared to be of same cell type and were OIF/NGF- positive. The abundance of OIF/NGF detected in the secretory acini was markedly less in the disseminate portion of the prostate gland (Figs. 3.1A and Fig 3.1C). In contrast to the body of the prostate, many acini did not contain OIF/NGF in the epithelium or in the lumen. In addition, where OIF/NGF was detected in the lumen in the disseminate prostate, many acini were devoid of OIF/NGF in the epithelium, and the number of cells containing OIF/NGF within a single acinus was lower compared to the body of the prostate (Fig. 3.1C) The distribution of secretory granules in the disseminate part, however, was similar to that of the body and were polarized to the apical region of the epithelial cells. The perinuclear distribution of OIF/NGF in several cells illustrates protein translation and storage into secretory vesicles (Fig. 3.1B and D). Another discernible difference between the body and disseminate regions of the prostate was the abundance of OIF/NGF detected in endothelium of blood vessels. The protein was detected in a greater proportion of arterial and venous vessels in the disseminate part than in the body of the prostate (Fig. 3.1D). Diffuse positive staining was found in the connective tissue between prostatic lobules that were closest to the acini; detection of OIF/NGF was lesser around vessels that were surrounded mostly by connective tissue and further away from acini.

The seromucus-secreting epithelium of the ampullae had no immuno-reaction to anti-OIF/NGF, while faint scattered staining was detected in the bulbourethral gland. OIF/NGF-

positive regions of bulbourethral glandular epithelium were few and found in the most apical region of the cells. Immuno-reactive OIF/NGF was not detected in the penile urethra, epididymis (head, body or tail) or the seminiferous tubule epithelium. Sperm within the testes and epididymides were also OIF/NGF negative. Myoid epithelial cells surrounding the seminiferous tubules were positive for OIF/NGF (++). A strong reaction was detected in the vascular endothelium and connective tissue fibers in the stroma and rete testes, but not in the interstitial cells.

Within the penis, the crura of the corpus cavernosum and corpus spongiosum were negative. The majority of positive staining was found in the connective tissue fibers of the tunica albuginea. Several blood vessels within the connective tissue had a weak to moderate immuno-reaction in the endothelium.

Table 3. 1 OIF/NGF immuno-reactivity in the male reproductive organs of llamas.

Tissue	Epithelium	Lumen	Connective tissue	Smooth muscle
Testis	-	-	++	-
Epididymis (head)	-	-	++	-
Epididymis (body)	-	-	++	-
Epididymis (tail)	-	-	+	-
Ductus deferens	-	-	++	-
Ampulla	-	-	+	-
Prostate (body)	+++	+++	+	-
Prostate (disseminate)	+++	++	+	-
Bulbourethral gland	±	±	+	-
Penis	-	-	++/+++	-

Relative OIF/NGF staining intensities were graded: +++ (very strong), ++ (strong), + (moderate to weak), ± (faint), - (absent).

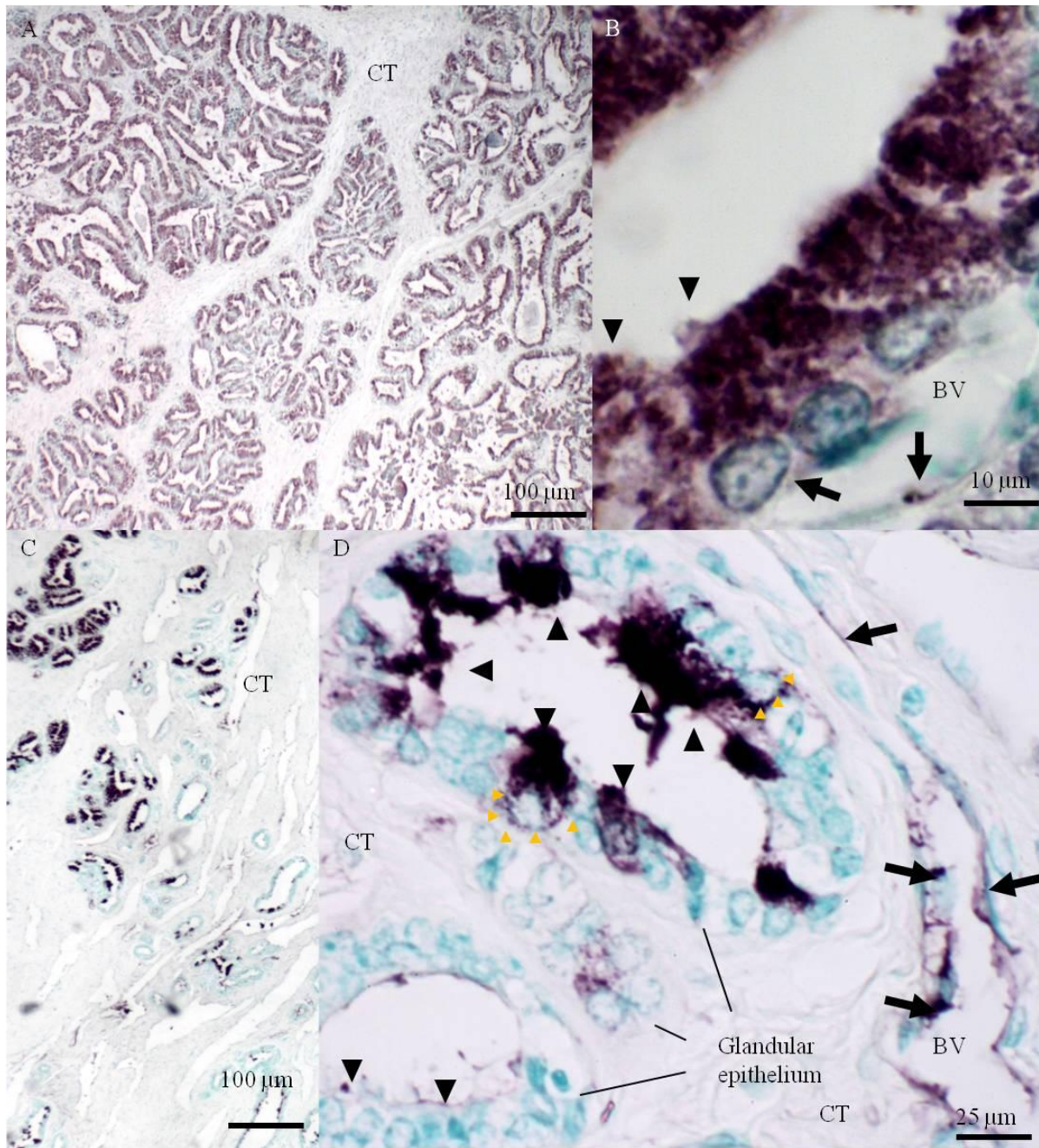


Figure 3. 1. Immuno-histochemical detection of OIF/NGF (purple) in the body (A and B) and disseminate (C and D) parts of the prostate gland of llamas. Cell nuclei are stained with methyl green. (A) OIF/NGF positive cells present in all acini of glandular epithelium. (B) Abundant OIF/NGF immuno-reactivity is detected in epithelial cells of a single prostatic acinus. Secretory granules are confined to the apical region of epithelial cells and secretion of OIF/NGF-containing granules into the lumen is evident (arrowheads). Little reactivity present in the endothelium or lumen of blood vessels (BV). (C) Relatively fewer OIF/NGF-positive acini in the disseminate prostate. (D) Detection of OIF/NGF immuno-reactivity in prostatic acini and blood vessel (BV). Presence of secreted OIF/NGF is evident in the lumen of glandular epithelium (arrowheads) and blood vessels (arrows). Yellow arrowheads indicate OIF/NGF distributed towards the basal portion and around the nucleus of the epithelial cells. ET: Epithelium; CT: Connective tissue. Scale bars as indicated.

3.4.2 OIF/NGF Immunolocalization- Rat

Immuno-staining for OIF/NGF was not detected in the epithelium of any of the accessory sex glands, but positive staining was observed in the lumen of blood vessels in the coagulating gland (Fig. 3. 2D). The connective tissue was OIF/NGF-positive within all organs but was variable in intensity (Table 3. 2). The appearance of the OIF/NGF-positive fibers in the connective tissue was similar among animals and was interpreted as nerve endings. Immuno-positive staining was detected in stereociliated cells of the head of the epididymis in scattered regions, but the reaction did not extend into the body or tail of the epididymis and was variable among individuals (Fig. 3. 2 A and B). Staining was repeated for the epididymal tissue, with similar results. OIF/NGF was not detected in the seminiferous tubule epithelium or in the lumen containing spermiated spermatazoa. Interestingly, intense staining was observed in interstitial cells in discrete aggregates throughout the testes. The connective tissue of the testis, ductus deferens, penis, coagulating gland displayed the greatest intensity of OIF/NGF-positive staining in the respective tissues.

Table 3. 2. OIF/NGF immuno-reactivity in the male reproductive organs of the rat.

Tissue	Epithelium	Lumen	Connective tissue	Smooth muscle
Testis	±	-	+++	-
Epididymis (head)	-	-/+++ *	+	-
Epididymis (body)	-	-	+	-
Epididymis (tail)	-	-	+	-
Ductus deferens	-	-	++	-
Vesicular gland	-	-	+	-
Coagulating gland	-	+++ **	+++	-
Dorsal prostate	-	-	+	-
Bulbourethral gland	-	-	-	-
Penis	-	-	+++	-

Relative OIF/NGF staining intensities were graded: +++ (very strong), ++ (strong), + (moderate to weak), ± (faint), - (absent), nd (not determined).

* Relative OIF/NGF staining intensities were different between rat 1 and rat 2.

** Detection found in lumen of blood vessels

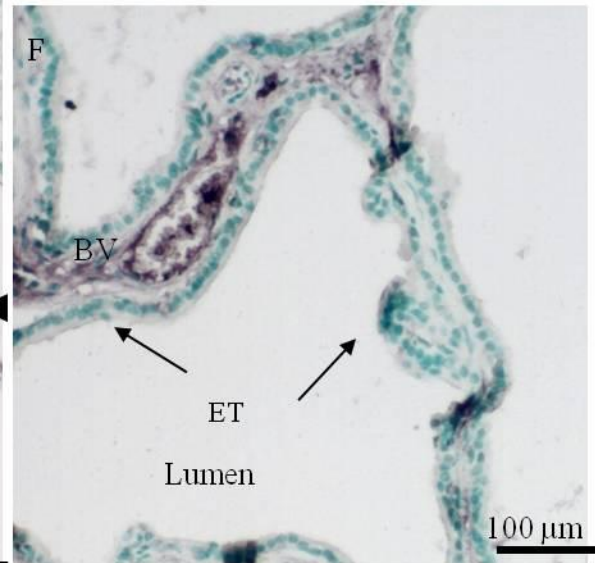
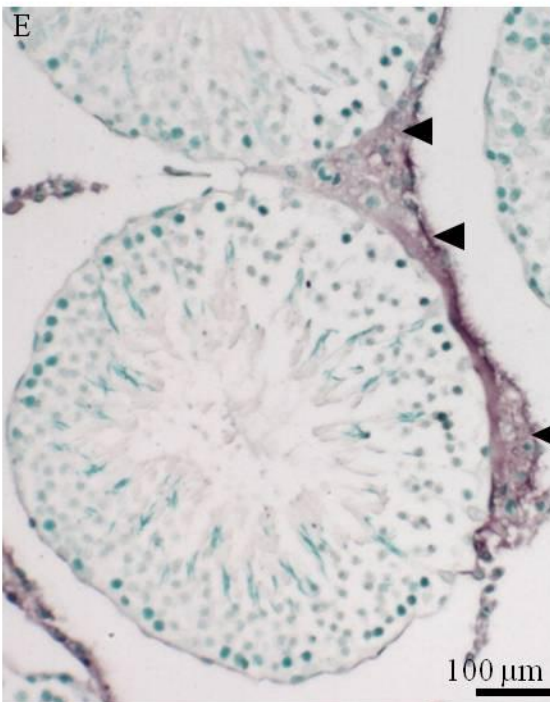
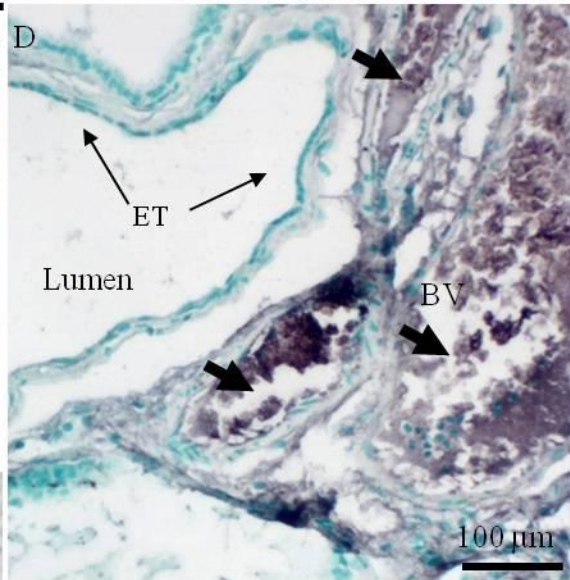
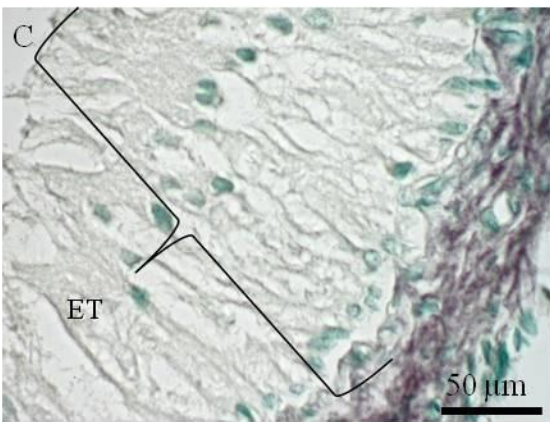
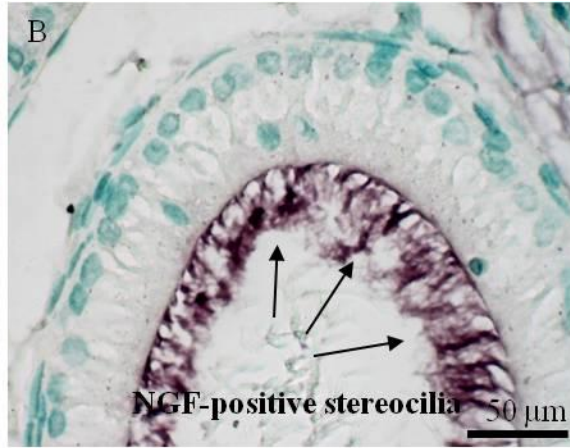
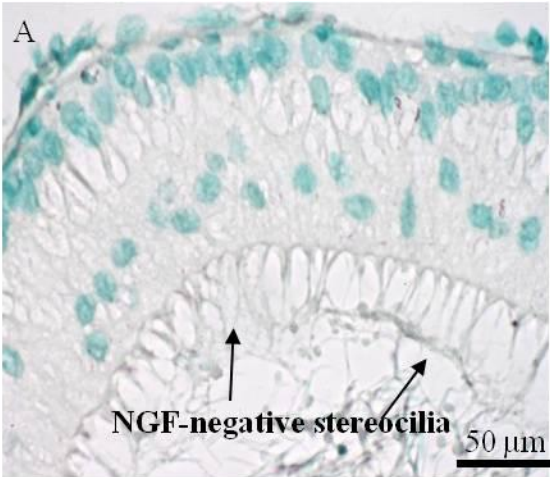


Figure 3. 2. OIF/NGF immuno-histochemical staining of the reproductive organs of the male rat. Differential staining was observed in the luminal surface of the head epididymis epithelium from Rat 1 (A) and Rat 2 (B). C, Ductus deferens epithelium exhibits strong OIF/NGF-immuno-reactivity in the CT. D, OIF/NGF-positive staining in blood vessel lumen of the coagulating gland. Strong (++++) reaction product is seen in interglandular blood vessels and connective tissue (arrows). E, Seminiferous tubules and OIF/NGF-positive reaction in interstitial tissue (arrowheads) . F, No OIF/NGF is detected in the glandular epithelium or lumen of the coagulating gland. But NGF-positive staining is visible in the connective tissue and lumen of blood vessels (arrows). ET: Epithelium; CT: Connective tissue. Scale bars are as indicated.

3.4.3 OIF/NGF Immunolocalization-Cattle

Tissue OIF/NGF immuno-reactivity in bulls is summarized in Table 3.3 and illustrated in Fig. 3.3. In contrast to llamas, OIF/NGF was detected within the epithelium of the ampullae. The degree of reactivity to OIF/NGF varied among glandular acini. For example, immuno-reaction was present in only a few cells of ampullary acini while in others it was present in most if not all the cells. Immuno-staining was strongest in the apical region of the glandular epithelium and was also detected in the lumen of the ampulla. Spermatozoa within the lumen of the ampulla, however, were OIF/NGF -negative. The distribution of OIF/NGF reactivity in the vesicular gland was similar to that in the ampulla. Positive immuno-reactivity was localized in the apical portion of the glandular epithelial cells and within the lumen. The peri-nuclear region of glandular epithelial cells was strongly positive in some and devoid of reaction in others within the same acinus. No immuno-reactivity was observed in the transitional epithelium lining the penile urethra or the endothelium of the sinuses of the corpus spongiosum and corpus cavernosum. The connective tissue surrounding the corpus spongiosum contained more OIF/NGF -positive fibers than the corpus cavernosum. OIF/NGF staining was evident in the smooth muscle layers surrounding the epididymis and blood vessels.

Weak staining was detected in the bulbourethral gland, and was confined to connective tissue and smooth muscle (Table 3.3). Immuno-reactivity to OIF/NGF was localized to the body of the prostate in glandular epithelial cells (Table 3.3).

In the testis, OIF/NGF immuno-reactivity was observed in the myoid epithelial cells around seminiferous tubules, spermatogonia, primary spermatocytes and sertoli cells. Round and elongated spermatids and interstitial cell cytoplasm were OIF/NGF negative. The vascular

endothelium in the testes and all regions of the epididymis were OIF/NGF positive. The nuclei in the epithelium of the tail displayed a weak immuno-reactive response.

Table 3. 3. OIF/NGF immuno-reactivity in the male reproductive organs of cattle.

Tissue	Epithelium	Lumen	Connective Tissue	Smooth Muscle
Testis	+++	-	++	+
Epididymis (head)	±	-	+++	++
Epididymis (body)	+	-	+++	+
Epididymis (tail)	+	-	+++	+ / ++
Ductus Deferens	±	+	+++	++
Ampulla	+++	+++	+++	+
Prostate (body)	++	-	+++	+
Vesicular gland	+++	+++	++	+
Bulbourethral gland	-	-	+	+
Penis	-	-	+++	+

Relative OIF/NGF staining intensities were graded: +++ (very strong), ++ (strong), + (moderate to weak), ± (faint), - (absent), nd (not determined).

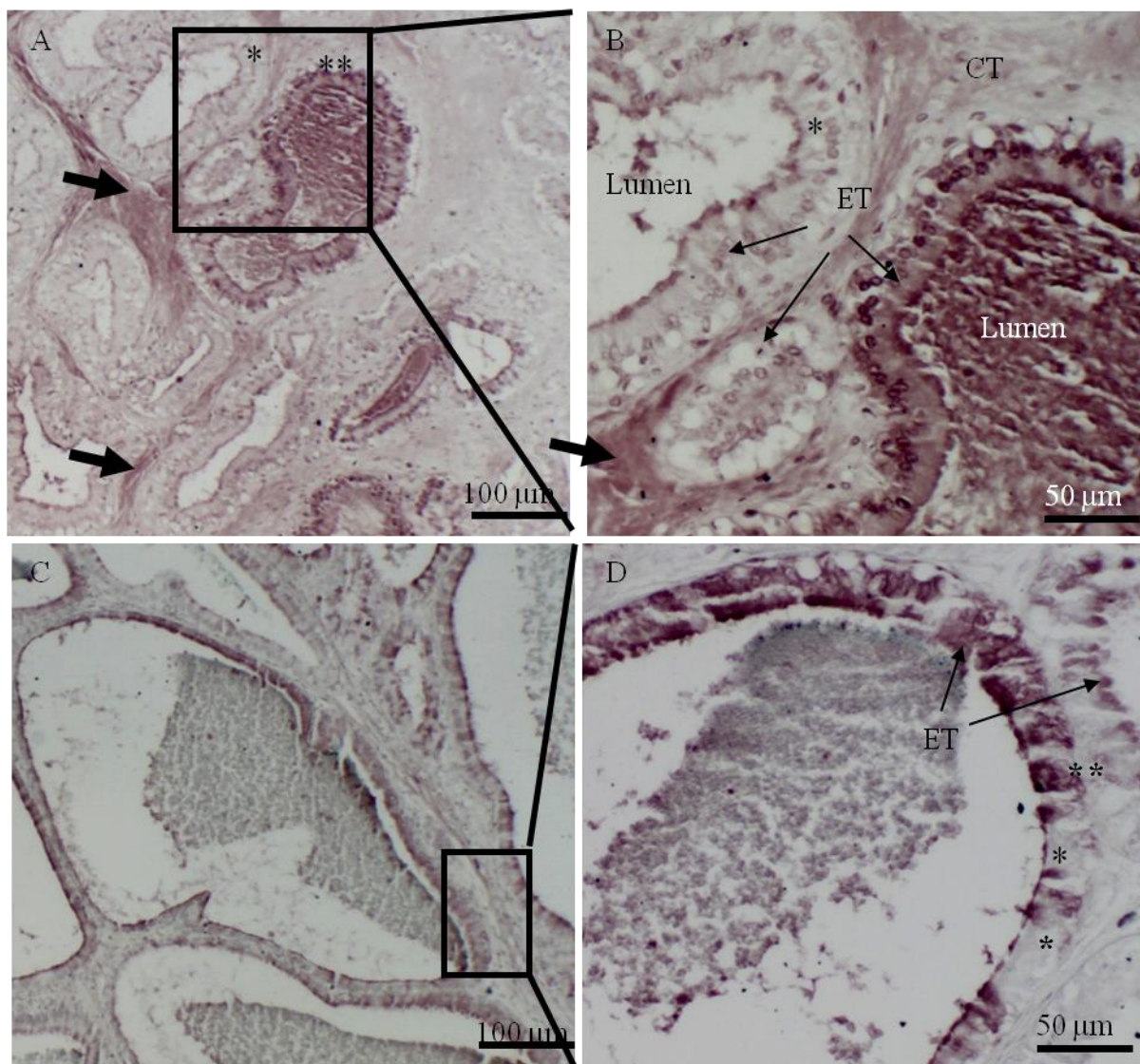


Figure 3. 3. OIF/NGF-positive immunoreactivity is detected (purple) in the ampullae (A and B) and vesicular glands (C and D) in cattle. A, Epithelium of glandular acini showing strong (**) and weak (*) immuno-staining staining in the ampullary gland. B, Magnified view of A, showing positive staining in the glandular epithelium (ET), lumen and connective tissue (CT). (Arrows) demark OIF/NGF detection in interlobular blood vessels. C, OIF/NGF-positive staining in the connective tissue and glandular epithelium of vesicular glands. D, Magnified view of C, showing OIF/NGF-positive staining in the apical region of epithelial cells and lumen. OIF/NGF-negative (*) and OIF/NGF-positive immuno-reactive epithelial cells are present within the same acini. ET: Epithelium; CT: Connective tissue. Scale bars as indicated.

3.4.4 OIF/NGF Immunolocalization- Bison

The ampulla and vesicular gland had the greatest immuno-reactive response among accessory glands in bison (Table 3. 4 and Fig. 3.4). Positive staining was detected in the connective tissue of the bulbourethral and prostate glands, but not in the glandular epithelium or lumen. While the connective tissue surrounding the glandular epithelium was OIF/NGF-positive, the nuclei of the cells were negative. The reactivity of the body of the prostate is similar to that observed in the disseminate prostate: no reactivity was seen in the glandular epithelium nor lumen. Although OIF/NGF immuno-reaction was detected in both bison bulls, the vesicular glands in one had more OIF/NGF in the epithelium and lumen than in the other where several acini were devoid of OIF/NGF in both the epithelium and the lumen (Fig. 3.4 A and B).

Ovulation-inducing factor /NGF positive sites were detected in the smooth muscle layers around the epididymal epithelium. Similarly, OIF/NGF was detected in the smooth muscle surrounding blood vessels as well as in the endothelium. Immuno-reactivity was not detected in the nuclei of smooth muscle or connective tissue cells. The nuclei of myoid cells found at the interface of the basement membrane and the lamina propria-submucosa expressed OIF/NGF strongly, along with other stellate-shaped nuclei in that region. The fibers within this region did not have uniform affinity to anti-OIF/NGF. Scattered fibers of similar diameter but variable lengths were strongly reactive in the stroma. These fibers were scarce in the connective tissue surrounding the glandular epithelium but were plentiful in the connective tissue more closely associated with the tunica muscularis. The arbitrary arrangement of these fibers as well as branching is consistent with terminal nerve tracts found in connective tissue. The nuclei of neurolemmocytes within peripheral nerve bundles as well as nerve axons contained OIF/NGF.

Table 3. 4. OIF/NGF immuno-reactivity in the male reproductive organs of bison.

Tissue	Epithelium	Lumen	Connective Tissue	Smooth Muscle
Testis	-	-	+++	-
Epididymis (head)	-	-	+++	++/+++
Epididymis (body)	±	-	+++	++/+++
Epididymis (tail)	±/+	-	+++	++/+++
Ductus Deferens	±	-	+++	++/+++
Ampulla	++/+++	+++	+++	-
Prostate (body)	-	-	+++	±
Prostate (disseminate)	-	-	+++	±
Vesicular gland	+/++	+++	+++	-
Bulbourethral gland	-	-	++	-
Penis	-	-	+++	++

Relative NGF staining intensities were graded: +++ (very strong), ++ (strong), + (moderate to weak), ± (faint), - (absent), nd (not determined).

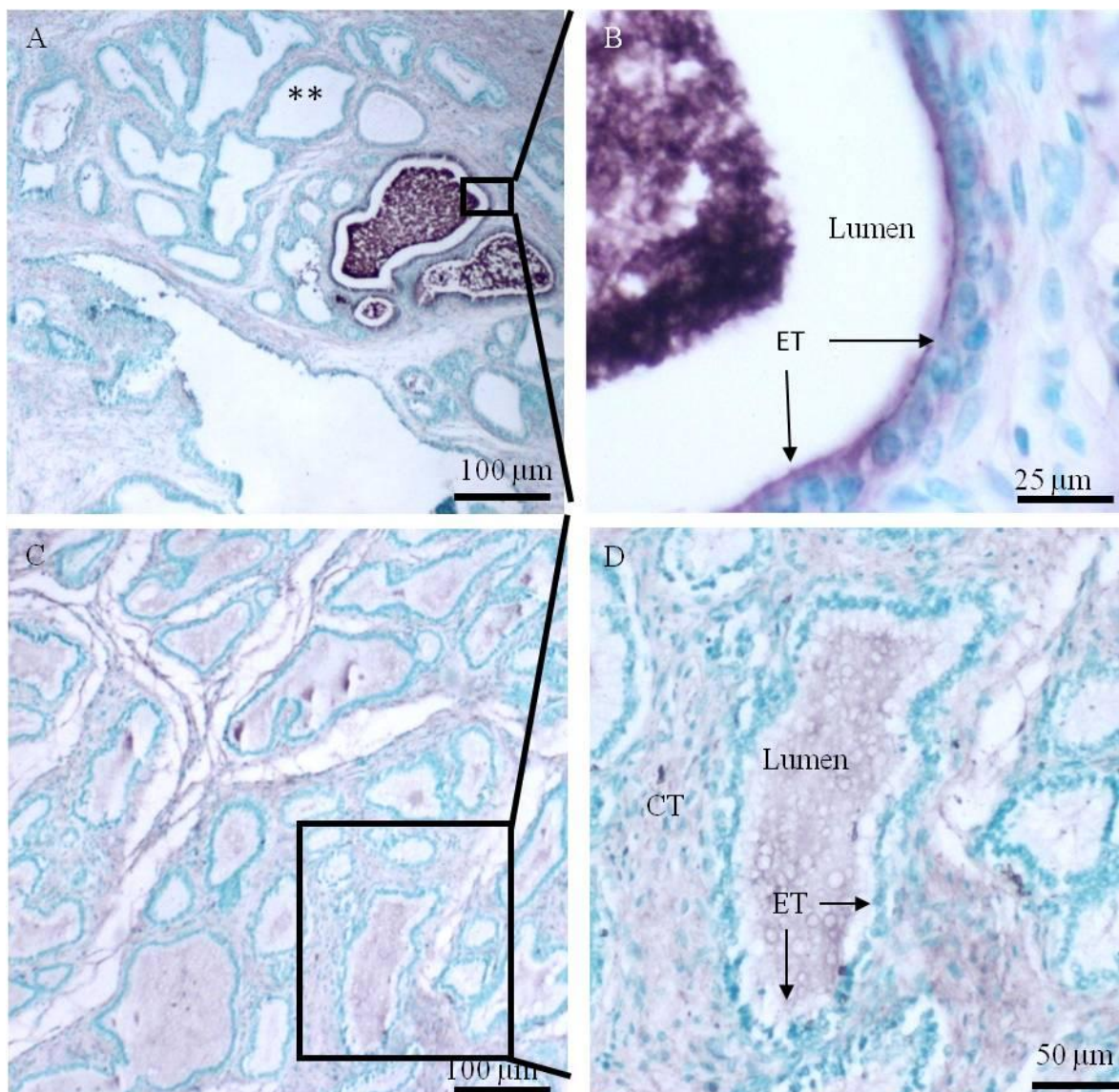


Figure 3. 4. OIF/NGF-positive immunoreactivity (purple) is detected in the ampullae (A and B) and vesicular glands (C and D) of bison. A, Acini showing positive (*) and negative (**) OIF/NGF staining in the ampullar gland. B, Magnified view of A, showing positive staining in the glandular epithelium (ET), lumen and connective tissue (CT). C, OIF/NGF-positive staining in the connective tissue and lumen of glandular lumen. D, Magnified view of C, showing OIF/NGF -positive staining in the connective tissue and lumen. Scale bars as indicated.

3.4.5 OIF/NGF Immunolocalization- Elk

Ovulation-inducing factor/NGF was detected in the lumen and glandular epithelium of the ampulla, but not in the glandular portion of any other accessory gland (Fig. 3.5). No immunoreactivity was observed in the seminiferous epithelium or interstitial cells of the testis. A strong positive reaction was detected in the connective tissue of all organs examined, and were interpreted as tissue innervation around blood vessels (Table 3. 5).

Table 3. 5. OIF/NGF immuno-reactivity in the male reproductive organs of North American Elk (Wapiti).

Tissue	Epithelium	Lumen	Connective	Smooth Muscle
Testis	-	-	+++	+
Epididymis (head)	-	-	++	±
Epididymis (body)	-	-	+++	+
Epididymis (tail)	-	-	+++	+
Ductus deferens	-	-	+++	+
Ampulla	++	+	++	-
Prostate gland (body)	-	-	++	-
Vesicular gland	-	-	+++	-
Bulbourethral gland	nd	nd	nd	nd

Relative OIF/NGF staining intensities were graded: +++ (very strong), ++ (strong), + (moderate to weak), ± (faint), - (absent), nd (not determined).

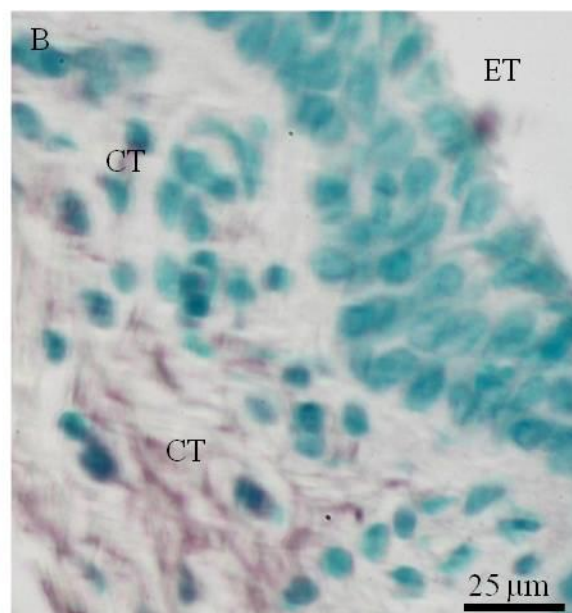
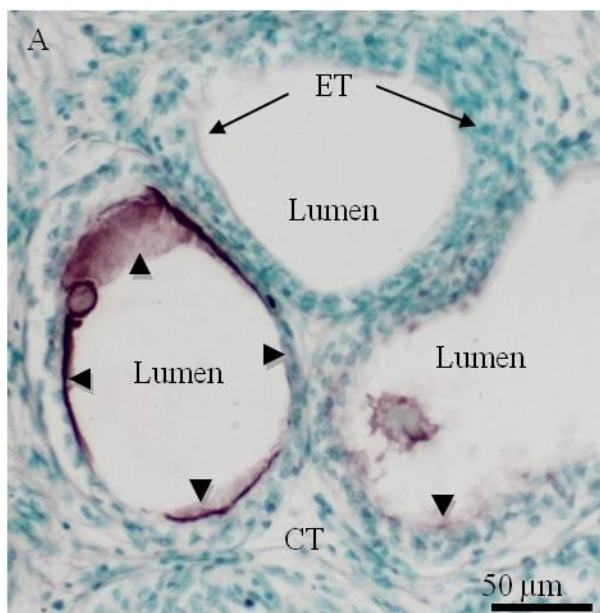


Figure 3. 5. OIF/NGF immuno-histochemical staining in elk ampullary gland. A, OIF/NGF - positive staining in the glandular epithelium and lumen of the ampullary gland. Arrowheads demark positive OIF/NGF reactions of varying intensities. B, Fibers within the connective tissue were stained by OIF/NGF antibody. Cellular nuclei were OIF/NGF negative in this section. ET: Epithelium; CT: Connective tissue. Scale bars as indicated.

3.4.6 OIF/NGF Immunolocalization- White-tailed Deer

Immuno-reactivity was detected in the epithelium and lumen of the prostate (Table 3. 6). The intensity of the detection in the epithelium was weak; several cell nuclei had a moderate reaction and reaction product within the glandular lumen was scattered and infrequent. The bulbourethral gland had a weak reaction and was confined to the nuclei within the epithelium. The nuclei of the cells of the epididymis were also positive. Strong immunoreactivity was detected in the smooth muscle cells surrounding the epididymal tubules.

Table 3. 6. NGF immuno-reactivity in the male reproductive organs of white-tailed deer.

Tissue	Epithelium	Lumen	Connective Tissue	Smooth Muscle
Testis	±	-	++	++
Epididymis (head)	+	-	++	++
Epididymis (body)	±	-	+++	±
Epididymis (tail)	±	-	+++	±
Ductus deferens	±	-	+++	±
Ampulla	±	+	+++	-
Prostate gland (body)	+ / +++	+	+++	±
Vesicular gland	-	-	+++	±
Bulbourethral gland	+	-	+	-

Relative NGF staining intensities were graded: +++ (very strong), ++ (strong), + (moderate to weak), ± (faint), - (absent).

3.4.7 Bioassays

The biological activity of OIF/NGF purified from llama/alpaca seminal plasma was confirmed by an *in vivo* llama bioassay and an *in vitro* PC₁₂ differentiation assay. All female llamas given purified OIF/NGF derived from pooled llama/alpaca seminal plasma ovulated (3/3; 100%) while none of those treated with saline ovulated (0/3; 0%). Thus, the purified OIF/NGF was deemed suitable for subsequent studies.

Differentiation of PC₁₂ cells *in vitro* was observed in wells treated with recombinant-NGF, purified OIF/NGF, and seminal plasma from llamas/alpacas and bulls (Table 3.7). Cells treated with seminal plasma from horses or pigs did not differentiate. The number of PC₁₂ cells induced to differentiate (dendrite growth) was greater in wells treated with OIF/NGF than in those treated with Cytochrome c ($P < 0.05$), and was decreased by the addition of anti-NGF (Table 3.8; $P < 0.05$).

Table 3. 7. Proportion of in vitro culture wells containing PC₁₂ cells that displayed dendrite growth (PC₁₂ differentiation assay for detecting NGF-like activity) after treatment with purified OIF or seminal plasma.

Treatment	Differentiated wells
rNGF	100% (3/3)
OIF/NGF	100% (3/3)
Llama/alpaca seminal plasma	100% (3/3)
Bull (bovine) seminal plasma	100% (3/3)
Equine seminal plasma	0% (0/3)
Porcine seminal plasma	0% (0/3)

Table 3. 8. Number of differentiated PC₁₂ cells per well (mean \pm SEM) in 10 microscopic fields at a magnification of 400x) after 4 days of *in vitro* culture with Cytochrome c or OIF/NGF, with or without NGF antibody.

	Cytochrome c	OIF/NGF
Without anti-NGF	0.4 \pm 0.13 ^a	17.5 \pm 5.53 ^b
With anti-NGF	0.1 \pm 0.03 ^a	0.2 \pm 0.06 ^a

^{abc} Values with different superscripts are different P<0.05.

3.4.8 Radioimmunoassay of OIF/NGF in semen

A total of 69 and 53 ejaculates were collected from llama/alpaca and cattle, respectively. Parallel displacement curves were observed with recombinant NGF, OIF/NGF, llama and bull seminal plasma (Fig. 3.6). Competition for antibody binding sites was observed in samples spiked with OIF/NGF but not with BSA. In addition, no displacement was found with either NT4 or BDNF neurotrophins, demonstrating the specificity of the assay. The lowest detectable limit was 10 µg/mL. The intra-assay coefficients of variation for the high (100 µg) and low references (25 µg) were 6 and 11%, respectively.

The total protein concentration was more than 40-fold greater in bull seminal plasma than llama/alpaca seminal plasma ($P < 0.0001$), but the OIF/NGF concentration was less than 1/10th that of llama/alpaca seminal plasma ($P < 0.05$; Table 3.9). No specific binding or parallel displacement was detected by radioimmunoassay with horse or pig seminal plasma. OIF/NGF represented 27.2% of total protein in llama/alpaca seminal plasma, and 0.2% of total protein in bull seminal plasma proteins ($P < 0.05$).

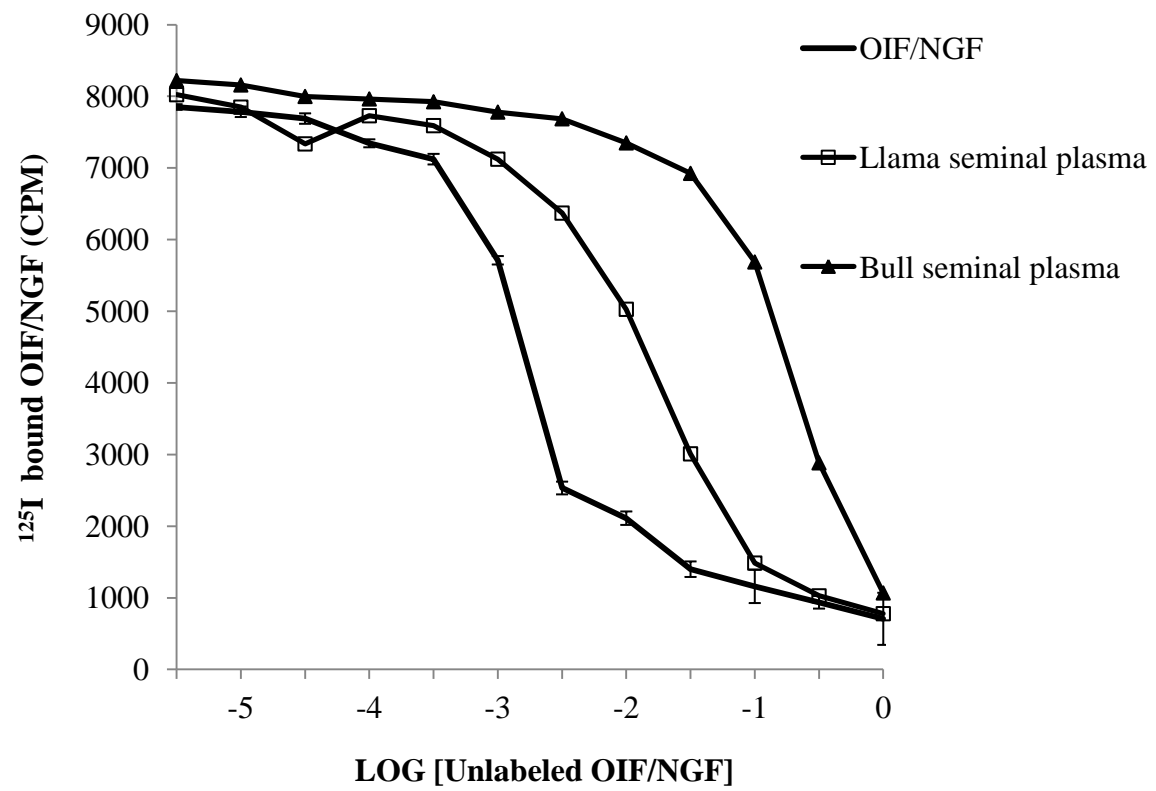


Figure 3. 6. Radioimmunoassay displacement curves among purified OIF/NGF from llama seminal plasma, llama and bull seminal plasma. The mean \pm SEM of 4 different curves represent the OIF/NGF. The log 0 concentration of OIF/NGF corresponded to 2.0 mg/mL. Diluted llama seminal plasma (1:1) was used as the Log 0 value for llama seminal plasma. Undiluted bull (cattle) seminal plasma was used as the Log 0 value. Parallel displacement curves are seen among the three samples.

Table 3. 9. Comparison of total protein and OIF/NGF concentration (mean \pm SEM) in llama/alpaca and bovine seminal plasma (SP).

Species	Total protein (mg/mL)	OIF/NGF (mg/mL)
Llama/alpaca SP (n=69)	4.4 \pm 0.59 ^a	1.2 \pm 0.21 ^a
Bovine SP (n=53)	48.6 \pm 4.84 ^b	0.10 \pm 0.03 ^b

^{ab} Means (\pm SEM) within columns are statistically different (P<0.05)

3.5 Discussion

Contrary to our expectation that the source of this widely conserved protein would be the widely conserved prostate gland, the principal source of OIF/NGF in seminal plasma was different among species. Of the six species examined in this study, the llama, bull (cattle) and white-tailed deer had immuno-reactivity to OIF/NGF in the mucosa of the prostate. Based on the presence of immuno-reactivity in both the glandular epithelium and glandular lumen, the prostate gland was the main source of seminal OIF/NGF in llamas and alpacas, but was the vesicular gland and ampullae in bovids (cattle and bison), the ampullae and the prostate in cervids (elk and white-tailed deer, respectively).

The extraordinary abundance of OIF/NGF immuno-reactivity in the llama prostate corroborates the high level found in seminal plasma. The perinuclear localization of OIF/NGF and the pattern of OIF/NGF storage in the llama prostate gland suggest that this protein is packaged in secretory vesicles. The diffuse pattern of OIF/NGF in connective tissue among all species is interpreted as tissue innervation [273].

An objective of this study was to further understand the importance of OIF/NGF in seminal plasma between animals categorized as spontaneous and induced ovulators. The differences in the abundance and distribution of OIF/NGF that were observed in the present study may relate to the biological importance of OIF/NGF in the respective species. Female llamas and alpacas ovulated in response to heterospecific seminal plasma but at a rate significantly lower than in response to camelid seminal plasma [42, 45, 54]. In contrast, neither pre-pubertal heifers nor mature cows ovulated in response to treatment with seminal plasma or purified OIF/NGF [55, 56]. However, bovine seminal plasma given to cows at the time of estrus resulted in more synchronous ovulations, and tended to stimulate a more rapid increase in

circulating progesterone concentration [56], similar to the luteotrophic effect seen previously in heifers (increase in CL diameter and plasma progesterone concentration) [55]. While OIF/NGF did not influence LH secretion and did not cause ovulation in cattle, treatment was associated with the release of FSH and earlier wave emergence [55]. Using an *in vitro* primary culture pituitary cells, it appears that the trkA receptor or the mechanism for LH secretion is conserved in mice[53], llamas and cattle[52]. In addition, seminal plasma has also been associated with an ovulatory role in mice [45], and estrous synchronization and luteogenesis in pigs [57, 274] [275].

The extraordinary difference in the abundance of OIF/NGF among species suggests a greater physiological importance in camelids. OIF/NGF represented approximately 27% of total protein in llama/alpaca ejaculates and less than 1% of total protein in bovine ejaculates. The difference in the OIF/NGF bioactivity of the seminal plasma of these species was confirmed by PC₁₂ differentiation assay. The difference in OIF/NGF concentration in the seminal plasma between these two species is consistent with the ovarian response *in vivo*. Seminal plasma from bulls induced ovulation in camelids at a rate comparable to that of llamas only when the amount of bovine semen given was increased to an estimated equivalent dose of OIF/NGF [42, 56]. This, and the finding that the endocrine and ovarian response to OIF/NGF is dose-dependent in camelids [49], provides rationale for the hypothesis that male fertility is positively related to the amount of OIF/NGF in the ejaculate.

Other than biological importance, the differences in the abundance of OIF/NGF in the ejaculate and in tissues in the present study may have been influenced by the method of semen collection and season of the year. Llama/alpaca ejaculates were collected by artificial vagina and phantom mount while bulls were collected by electroejaculation. Protein configuration within

ejaculates can be altered by collection method. A comparison of the seminal plasma proteome in cats where semen was collected by electroejaculation or urethral catheterization found that while total protein concentrations were not different, two proteins were absent and the abundance of three different proteins were higher in samples collected by electroejaculation [276]. Similar results were found in Guirra rams; the concentration of two proteins were higher and one protein absent in samples collected by electroejaculation in comparison to samples collected by artificial vagina [277]. These observations confirm that the ejaculation mechanism is somewhat altered with electroejaculation. In addition, semen was collected from llamas/alpacas during the summer months while semen was collected from bulls during winter and spring months. Although camelids and cattle are not seasonal breeders, two dimensional electrophoresis of ram seminal plasma identified at least 16 proteins which quantitatively changed in relation to the season [278].

Interestingly, no OIF/NGF was detected in the seminal plasma of horses or pigs by either PC₁₂ assay or by radioimmunoassay. In a recent study involving multidimensional chromatography to compare seminal plasma proteins among species, OIF/NGF was found in the seminal plasma of bulls, stallions and camelids but was absent in boars [43]. This is in contrast to the results of a previous study in which boar seminal plasma given intramuscularly induced ovulation in 18% (3/17) llamas [45]. In the latter study, however, a biological response was observed using a volume that was five-times greater than that of llama seminal plasma. Histological confirmation of OIF/NGF in horse and pig male reproductive system was not done in this study. Since OIF/NGF were not measureable in the seminal plasma of horses and pigs but can still induce an ovulatory response in camelids, suggests that the quantity of this protein is

very low, below the detection limit of our assay, or perhaps there are other factors in addition to OIF/NGF in seminal plasma than can trigger an ovulatory repose in camelids.

OIF/NGF in testis and epididymides, and sperm: In this study, a strong reaction was detected in the seminiferous epithelium of cattle but not in llamas. In an earlier study, NGF immuno-reactivity was detected in the perinuclear cytoplasm of germ cells from prepubertal and pubertal alpacas [250]. However, it was mentioned that the immuno-detection of NGF had decreased in mature 24-month old alpacas in comparison to prepubertal 12-month old alpacas[250]. In our study, all animals used were pubertal. The detection of NGF in nuclei in the seminiferous epithelium supports that NGF is a mitogenic factor[279, 280] or transcription factor [281, 282]. NGF stimulated meiotic DNA synthesis of preleptotene spermatocytes cultured in vitro culture in a dose-dependent manner [283] and also rapidly induced the expression of genes involved germ cell differentiation[284, 285].

One of the most interesting observations from this study was the abundance of the OIF/NGF present within the vascular endothelium circulatory system. Fighting episodes, male aggression or stress in mice caused a dramatic increase in NGF levels in circulation [209, 286, 287] and in both mRNA and protein levels in the hypothalamus [268]. The administration of testosterone propionate did not elevate NGF levels in nonaggressive males but augmented serum NGF levels in aggressive males [286]. The salivary glands and not the male accessory genital glands were the source of NGF secretion in mice related to aggressive episodes since no change in NGF concentration was observed once the submandibular-sublingual salivary glands were removed [209, 286]. However, the detection of OIF/NGF in the vascular endothelium in male reproductive tissue suggests there are additional sources of OIF/NGF found in circulation. Uptake into circulation suggests that endogenous NGF has a direct effect on spermatogenesis.

The presence of the high affinity (trkA) and low affinity (p75) NGF receptors in male reproductive organs and sperm suggest a role for NGF on sperm function [249, 251, 288, 289]. Correspondingly, the OIF/NGF receptors have been localized in the testes and their expression changes in relation to the hormonal state and stage of the cycle of the seminiferous epithelium [283]. In rats, the low affinity OIF/NGF receptor was detected in the plasma membrane of Sertoli cells and its distribution to either the apical or basal regions of the cell was dependent on the stage of the cell cycle [283]. The expression of the p75 receptor in Sertoli cells was detected only in stages VII and VIII of the seminiferous epithelium cycle which corresponds to the stages of meiosis [283]. During these stages, the p75 receptor is down regulated by testosterone. Testosterone has also been shown to downregulate p75 expression in certain regions of the brain [290]. Rats infused with NGF directly into the testes resulted in an increase of androgen-binding protein mRNA in Sertoli cells [291].

In most cases, the distribution of OIF/NGF was similar between animals among species. One remarkable difference was observed in the head of the epididymis of the rat. The detection of OIF/NGF in proximity to stereocilia of rat 2, suggests the absorption of OIF/NGF into the epithelium. The weak detection of OIF/NGF in developing germ cells was different from what was reported previously in rat where OIF/NGF was detected in germ cells of all stages of spermatogenesis as well as in mature spermatazoa [247, 283]. No reaction, however, was observed in Sertoli or Leydig cells [210]. In rats, OIF/NGF was detected in the cytoplasm of developing germ cells but was absent in Sertoli cells and was found in very few interstitial cells. The weak reaction of OIF/NGF in the cytoplasm of developing germ cells may be the result of dose or antibody affinity. In the present study, a dilution of primary antibody of 1:200 was used to visualize OIF/NGF immuno-reactivity because no reaction was observed with the dilution

used for the other tissue (1:400). In one study, three different antibodies were used to screen OIF/NGF in male mice genitalia and immuno-reactivity was detected with only one of the antibodies [210]. The same antibody was used for all other tissues from all species in this experiment to allow direct comparison of staining intensities. In addition, *in vitro* studies to confirm antibody specificity of binding to OIF/NGF, detection of monomer, dimer and prohormone forms, and the suppression of OIF/NGF neurite growth from PC₁₂ cells were conducted in our laboratory.

We conclude that the prostate gland is the primary source of OIF/NGF in the ejaculates of llamas and white-tailed deer but not in other species. The purpose for OIF/NGF in other species is unclear and needs further investigation. The differences observed between animals, such as rats and bison, indicate that OIF/NGF abundance differs among animals of the same species. Use of the methodology developed in the present study will enable testing of the hypothesis that male fertility is positively related to the amount of OIF/NGF in the ejaculate.

3. 6 Acknowledgements

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4.0 THE RELATIONSHIP BETWEEN OVULATION-INDUCING FACTOR/NERVE GROWTH FACTOR (OIF/NGF) AND MALE REPRODUCTIVE PHYSIOGNOMIES IN LLAMAS AND ALPACAS

4.1 Abstract

Ovulation in camelids is initiated by the seminal plasma protein, ovulation inducing factor/nerve growth factor (OIF/NGF). The intramuscular administration of OIF/NGF has demonstrated that this protein elicits an ovulatory response in a dose-dependent manner. However, the abundance of this protein has not been quantified in camelids to ascertain whether the concentration is variable among males. We hypothesize that the abundance of OIF/NGF differs among males and that OIF/NGF concentration is correlated with normozoospermic semen samples. The objectives of the present study were to determine if OIF/NGF quantity in seminal plasma is associated with male reproductive parameters and to elucidate the variability of OIF/NGF levels in alpaca ejaculates. The experiment was conducted at Quimsachata research station in Peru and ejaculates ($n \geq 3/\text{animal}$) from alpacas ($n=47$) were collected. Semen was collected by artificial vagina and the duration of breeding, sperm concentration, semen pH and volume, total protein and OIF/NGF concentrations were recorded. Total protein concentrations were quantified by Bradford's assay and OIF/NGF concentrations were quantified by radioimmunoassay. Areas of the testes, bulbourethral and prostate glands were estimated by calipers (testes) and transrectal ultrasonography (bulbourethral and prostate). Although OIF/NGF concentration was not related to sperm motility, vitality or morphology, there was a positive association with sperm concentration. The total quantity of OIF/NGF per ejaculate, however,

was not associated with any semen parameter and was related to prostate area, duration of mounting and semen volume.

4.2 Introduction

Ovulation in South American camelids occurs naturally following mating. The current hypothesis suggests the male ejaculate contains an abundant protein that is absorbed into circulation via penile abrasion of the uterine lining, stimulating LH release and initiating the ovulatory mechanism [48, 171, 239, 292]. This protein was originally referred to as ovulation-inducing factor (OIF) [48, 246], but recent studies have revealed that this protein is identical in sequence and structure to the neurotrophin, nerve growth factor (NGF) [39, 40]. For clarity and continuity, the factor isolated and purified from camelid seminal plasma will be referred to herein as OIF/NGF. The existence of OIF/NGF or an ovulatory effect of seminal plasma have been documented in the ejaculates of several species including camelids [39-41], bulls [44, 293], koala [294], boars [45, 295], stallions [45], mice [45] and men [46].

The isolation of OIF/NGF from camelid seminal plasma has allowed the ability to directly study the effect of OIF/NGF on female physiology. The administration of OIF/NGF to llamas or alpacas, increases circulating levels of LH within 15 minutes. In comparison to GnRH, the LH surge lasts longer with OIF/NGF administration and the resulting CLs formed, are more luteotrophic [48, 52, 171, 239, 292]. Ovulation rate, LH and progesterone concentration in circulation and CL lifespan are influenced by the dose of OIF/NGF administered when given intramuscularly [49, 52]. It remains to be elucidated whether the abundance of OIF/NGF varies among males and if this difference is manifested in an attenuated ovulatory response in females.

The primary source of OIF/NGF is the prostate gland in camelids (Chapter 3, Bogle Thesis, 2015). The ability of vasectomized alpacas and llamas to induce ovulation in females clearly demonstrate that sperm and factors derived from the epididymis and testis are not

required [12]. However, the presence of the high affinity receptor (trkA) and low affinity receptor (p75) for NGF in male reproductive organs and sperm suggest a role for NGF in sperm function [249, 251, 288, 289]. In support of this notion, the addition of exogenous NGF to golden hamster semen increased sperm motility in a dose- and time-dependent manner, and increased the percentage of sperm that underwent the acrosome reaction [248]. The addition of NGF to human ejaculates increased motility in progressively motile sperm [255] and in bovine, exogenous NGF increased sperm viability [249].

Few reports are available on the concentration of NGF in mammalian semen or its relationship with sperm morphology and function. In one study, NGF concentration in human seminal plasma was quantified using an enzyme-linked immunosorbent assay with values that ranged from 0.13 to 1.4 ng/mL [296]. The variation in NGF concentration in seminal plasma were not attributed to male fertility as no difference were observed among fertile, oligoasthenozoospermic or asthenozoospermic men (0.82 ± 0.1 ng/mL, 0.68 ± 0.2 ng/mL and 0.79 ± 0.1 ng/mL, respectively) [46]. Nevertheless, the associations between OIF/NGF concentration or total abundance in an ejaculation with male reproductive parameters has not been elucidated in alpacas. We hypothesize that ejaculates containing greater quantity of OIF/NGF will also have better semen quality in terms of sperm concentration, motility and viability. The objectives of the present study were to 1) determine OIF/NGF concentrations in alpaca semen and 2) to evaluate the relationships between OIF/NGF concentration/total abundance in the ejaculate with male reproductive organs and semen characteristics.

4.3 Materials and Methods

4.3.1 OIF/NGF radioimmunoassay

Total protein concentration of seminal plasma was quantified using Bradford's method according to the manufacturer's directions [272] (Bio-Rad Laboratories, Mississauga, Ontario, Canada), and OIF/NGF concentration was measured by validated double-antibody radioimmunoassay (Bogle et al., 2014, Chapter 3 PhD thesis). Purified, biologically active OIF/NGF isolated from pooled llama/alpaca seminal plasma was labeled with ^{125}I by the chloramine-T method [270]. The primary antibody was a rabbit anti-human NGF polyclonal antibody (Santa Cruz Biotechnology, Dallas Texas, USA) and was used at a dilution of 1:1000. The secondary antibody was goat serum raised against rabbit globulins (Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada [271]. Seminal plasma samples were diluted with PBS to a concentration that was within the range of assay detection. Values were then corrected for their dilution factor. Samples were assayed in duplicate with a standard curve ranging from 0 to 200 $\mu\text{g/mL}$. The lowest detectable limit of the assay was 10 $\mu\text{g/mL}$. Within each experiment, all seminal plasma samples were measured in a single assay to remove interassay variation. The intra-assay coefficient of variation for the low (25 μg) and high (100 μg) references were 10% and 6%, respectively.

4.3.2 Semen collection

The study was conducted during January and February (rainy season) at the Quimsachata Research Station in the department of Puno, Peru (15°S, 71°W, and 4500 m above sea level). Males were chosen from a herd of about 100 based on their ability to serve the phantom mount.

Males (n=39 Huacaya and n=8 Suri) ranged from 4-14 years of age, 45-74 kg body weight, and were maintained on natural pasture throughout the study.

Animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

Males were trained to use the phantom mount for a three-week period before sample collections. Acceptance of the phantom mount was encouraged by applying freshly collected vaginal swabs of urine from receptive females to the phantom mount and orifice of the artificial vagina. In some instances, a live receptive female was placed beside the phantom mount to train the male to associate it with copulation. Once the association was established, a live female was no longer used. To facilitate the workload, males were divided into one of three collection periods (first group: n=16 males; second group: n=16; third group: n=15). Semen samples from males within groups were collected on the same day using four separate phantom mounts. Ejaculates were collected twice from each male on each of 6 collection days, with at least five hours of rest between collections on a given day, and at least one full day of rest between collection days. The duration of mounting and time of day were recorded for each collection period. Males were allowed six independent opportunities to service a mount. A successful collection was defined as the ability of a male to mount and give a sample that contained semen.

4.3.3 Analysis of ejaculates

Ejaculates were evaluated for semen volume, froth volume, viscosity, pH, sperm concentration, motility, viability and morphology. Semen volume and froth volume were determined directly from the collecting tube (BD Biosciences, San Jose, California). Semen

volume was defined as the liquid portion of the aliquot at the bottom of the tube; the froth volume was the foamy portion that was not liquefied. Viscosity was determined by aspirating semen through an 18 Ga needle, placing a drop onto a glass slide and pulling the semen mass upwards. The distance from the initial drop of semen on the slide to the maximum distance before the semen retracted back to the slide was taken as the viscosity measurement. Samples in which viscosity resembled that of water were given a measurement of 0 mm. Semen pH was measured using a pH-meter (Hanna Instruments, Premier Farnell UK Limited, Leeds, United Kingdom) and confirmed with a litmus paper (Whatman/GE Healthcare, Mississauga, Canada).

Semen concentration was estimated using a Neubauer hemocytometer. Semen was homogenized using a transfer pipette and a 10 μ L aliquot was aspirated and placed on each side of the hemocytometer chamber. The mean number of sperm counted in both chambers was used as the concentration. Samples where no spermatozoa were observed in fresh preparations were considered azoospermic [297].

Total sperm motility was determined by observing a minimum of 10 fields at random using a light microscope under 400 x. Sperm viability and morphology were evaluated using Eosin-Nigrosin stain. A minimum of 20 fields and 200 sperm per sample were assessed for both live-dead and sperm morphology counts under 1000x with immersion oil. The sperm were examined for defects of the head, mid piece, principle piece and acrosome. Samples with a low sperm concentration (where 200 sperm were not counted) were excluded from morphology analysis. Analysis of all ejaculates was done by the same operator.

Ejaculates were processed according to previous studies [45, 48] with slight modification: ejaculates were centrifuged first at 500 x g for 15 minutes to separate plasma from spermatozoa without breaking/disrupting cell membranes. The supernatant was re-centrifuged at 1500 x g for

15 minutes. An aliquot of seminal plasma was viewed under a light microscope to ensure that no spermatozoa remained in the sample. If sperm were present, samples were re-centrifuged at 1500 x g for 15 minutes until no sperm were observed under a light microscope. The seminal plasma was stored in liquid nitrogen until assay.

4.3.4 Morphometry of testis and accessory sex glands

Testis area (mm²) was calculated by vernier calipers. The maximum height and width of the right and left lobes of the body of the prostate (prostate gland area: mm²), and the maximum height and width of the right and left bulbourethral glands (bulbourethral gland area mm²) were estimated by transrectal ultrasonography (7.5 MHz linear array transducer, MyLab Five, Esaote, Italy: Fig. 4. 1). The area (A) formula for an ellipse was used to estimate the area of each accessory gland: $A = \pi(\text{radius of long axis})(\text{radius of short axis})$. The areas of the left and right lobes of the prostate gland and left and right bulbourethral glands were summed, respectively, to provide a total area measurement. Accessory sex gland, testis and a blood sample for testosterone measurement were taken once, on the same day, during the collection period.

4.3.5 Testosterone concentration

One blood sample was collected from each male during the collection period into heparinized tubes by jugular venipuncture (BD Biosciences, Mississauga, Canada) and centrifuged at 1500 x g for 15 minutes. The plasma was aspirated and stored at -20°C until testosterone assay. Testosterone concentrations were measured using a commercial radioimmunoassay kit (Siemens Medical Solutions USA, Inc., Malvern, PA, USA). All samples

were measured in a single assay. The intra-assay coefficient of variation for the low, medium and high references were 14%, 6% and 5%, respectively.

4.3.6 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System package (SAS, Learning Edition, version 4.1, SAS Institute Inc., Cary, NC, USA, 2006). The concentration of OIF/NGF and total OIF/NGF among males were compared by Kruskal-Wallis nonparametric test. The degree of heterogeneity of OIF/NGF concentration and total abundance within males were assessed by Kruskal Wallis nonparametric test of the absolute values of residuals from the mean. Stepwise regression was used to build a prediction model for the total OIF/NGF per ejaculate.

Spearman's rank correlation was used to determine the relationship between variables. Student's t-test was used to compare differences in semen parameters between the first and second ejaculates on a given collection day. All data are reported as mean \pm SEM and $P \leq 0.05$ were considered significant, whereas $P > 0.05$ but ≤ 0.10 were considered trends approaching significance.

4.4 Results

4.4.1 Relationship between OIF/NGF concentration and other characteristics of the ejaculate

A total of 316 collections were attempted and a total of 243 ejaculates were collected and analyzed. Failed collections (n=73) were due to 1) inclement weather (38/73), lack of libido (26/73), failed ejaculation (6/73) and urine contamination (3/73). The means (\pm SEM) for all

endpoints measured are shown in Tables 4.1-4.3. Only 9.1% (22 of 243) of ejaculates did not contain sperm. Almost all ejaculates had froth (241/243; 99%). The only endpoint which correlated with amount of froth was mounting duration ($\rho=0.35$; $P=0.001$). An overview of male reproductive endpoints and sperm morphology are shown in Tables 4.1- 4.3 and Fig.4.1-4.3.

The mean concentration of OIF/NGF per ejaculate was 6.1 ± 0.7 mg/mL and the total OIF/NGF per ejaculate was 7.0 ± 1.1 mg (n=153). Total protein concentration and protein content per ejaculate were 11.6 ± 1.4 mg/mL and 15.5 ± 1.9 mg, respectively. The amount of OIF/NGF per ejaculate and per mL of seminal plasma were different among males ($P=0.03$). The heterogeneity in both OIF/NGF concentration ($P<0.0001$) and total abundance per ejaculate ($P<0.0001$) was reflected in the analysis of absolute residuals for each male. The majority of the ejaculates collected contained more than 250 μ g of OIF/NGF (Table 4. 4).

Correlations between all endpoints are shown in Table 4.5. OIF/NGF concentration was positively correlated with sperm concentration and total prostate area and negatively with semen volume, viscosity, pH and bulbourethral area. Total OIF/NGF per ejaculate was positively correlated with OIF/NGF concentration, prostate area, duration of mounting and semen volume (Table 4. 5). The application of stepwise multiple regression analysis revealed 3 independent variables, namely OIF/NGF concentration, semen volume and prostate area to predict the amount of total OIF/NGF per ejaculate with 56% ($R^2=0.56$) total variance explained (Table 4. 6).

Overall, first and second collections on a given day did not differ in OIF/NGF concentration (6.3 ± 1.0 vs. 5.1 ± 1.0 mg/mL; $P=0.43$), total OIF/NGF per ejaculate (7.7 ± 1.6 vs. 5.4 ± 1.3 mg; $P=0.33$), semen viscosity (3.0 ± 0.3 vs. 3.2 ± 0.3 mm; $P=0.58$), sperm concentration (89.3 ± 14 vs. $62.1 \pm 9.2 \times 10^6$ sperm/mL; $P=0.16$), or percentage of motile sperm

(39.3 ± 2.4 vs. 40.0 ± 3.1 ; $P=0.86$). Similarly, sperm morphology was not different. The duration of collection (24.8 ± 0.6 vs. 22.3 ± 0.6 minutes; $P=0.01$), semen volume (1.9 ± 0.2 vs. 1.1 ± 0.2 mL; $P=0.03$), and pH (7.6 ± 0.04 vs. 7.5 ± 0.05 ; $P=0.03$) were greater in the first ejaculate of the day than in the second.

Sperm morphology was not associated with OIF/NGF concentration or total amount in the ejaculate (Table 4.5). Abnormal head shape was most the commonly observed morphologic defect in ejaculates, followed by midpiece defects (Table 4. 3; Figs. 4. 3 and 4. 4). Head defects were higher in older males ($\rho=0.29$; $P=0.007$) with low sperm concentration ($\rho=-0.20$; $P=0.01$) lower acidic-neutral semen pH ($\rho=0.27$; $P=0.01$). Midpiece abnormalities were negatively associated with sperm motility ($\rho = -0.23$; $P=0.04$).

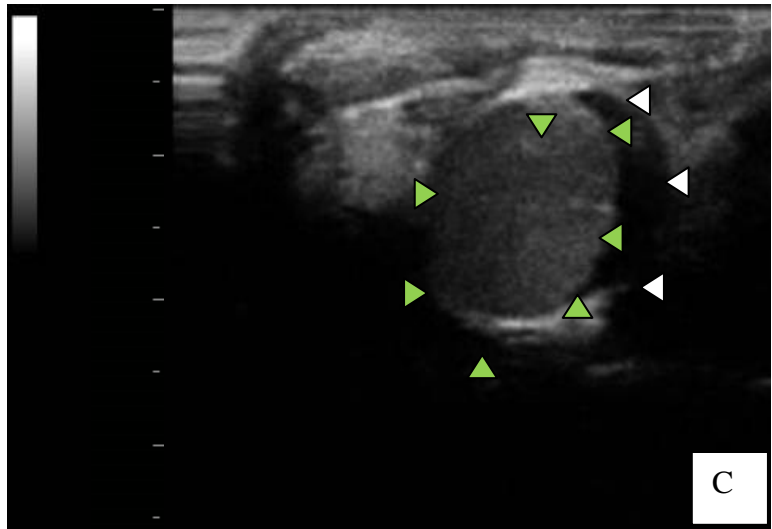
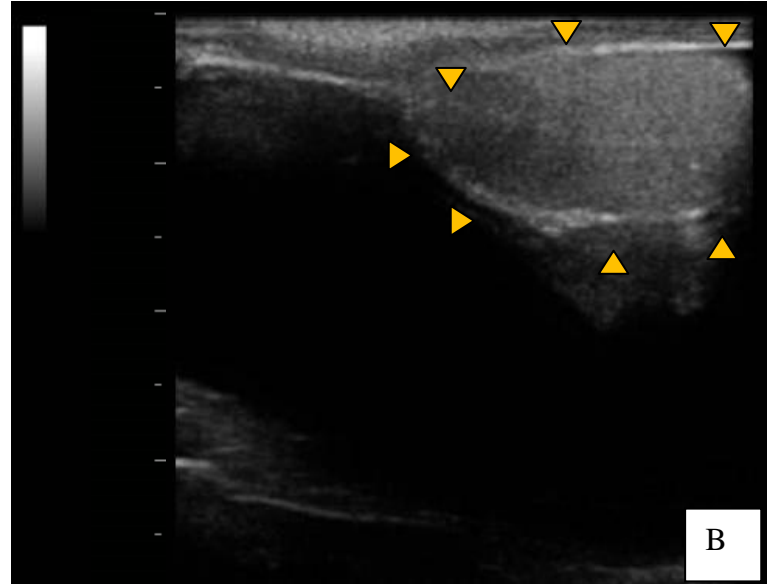
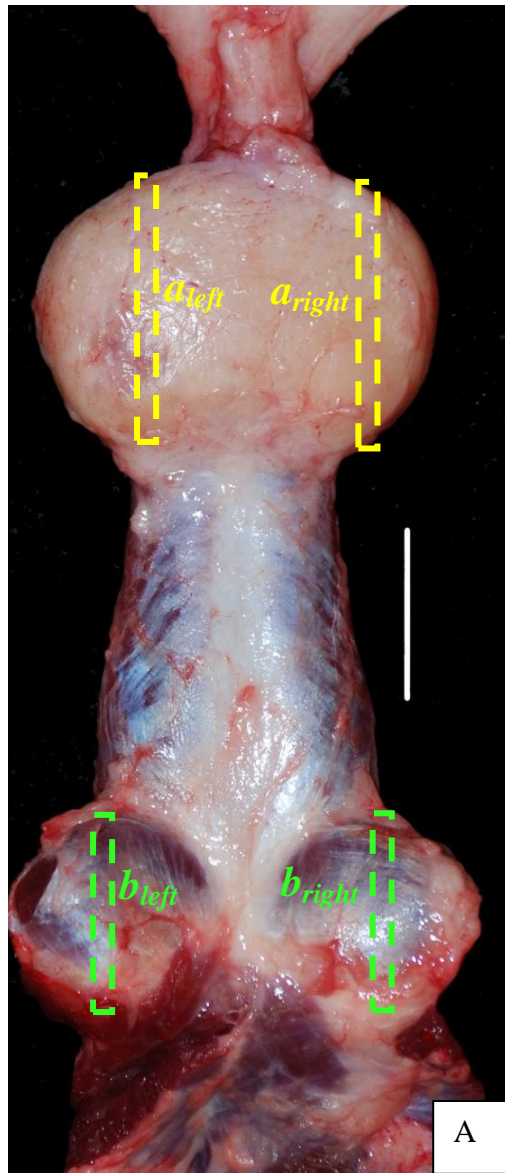


Figure 4. 1. Gross and ultrasonographic morphology of the pelvic urethra and accessory glands of a male alpaca. A, *Ex situ* dissection of pelvic urethra (dorsal view) showing the left and right lobes of the compact portion of the prostate (*Pr*) and the left and right lobes of the bulbourethral gland (*Bu*). Ultrasound image of a sagittal section of the prostate gland (B) and bulbourethral gland (C) along the plane indicated by the dashed lines. *Arrowheads* outline the glandular tissue. Note the crescent-shaped anechoic region along the caudal aspect of the bulbourethral gland. Distance between major scale lines is 1 cm. Ultrasound images taken using a transrectal 7.5 MHz probe.

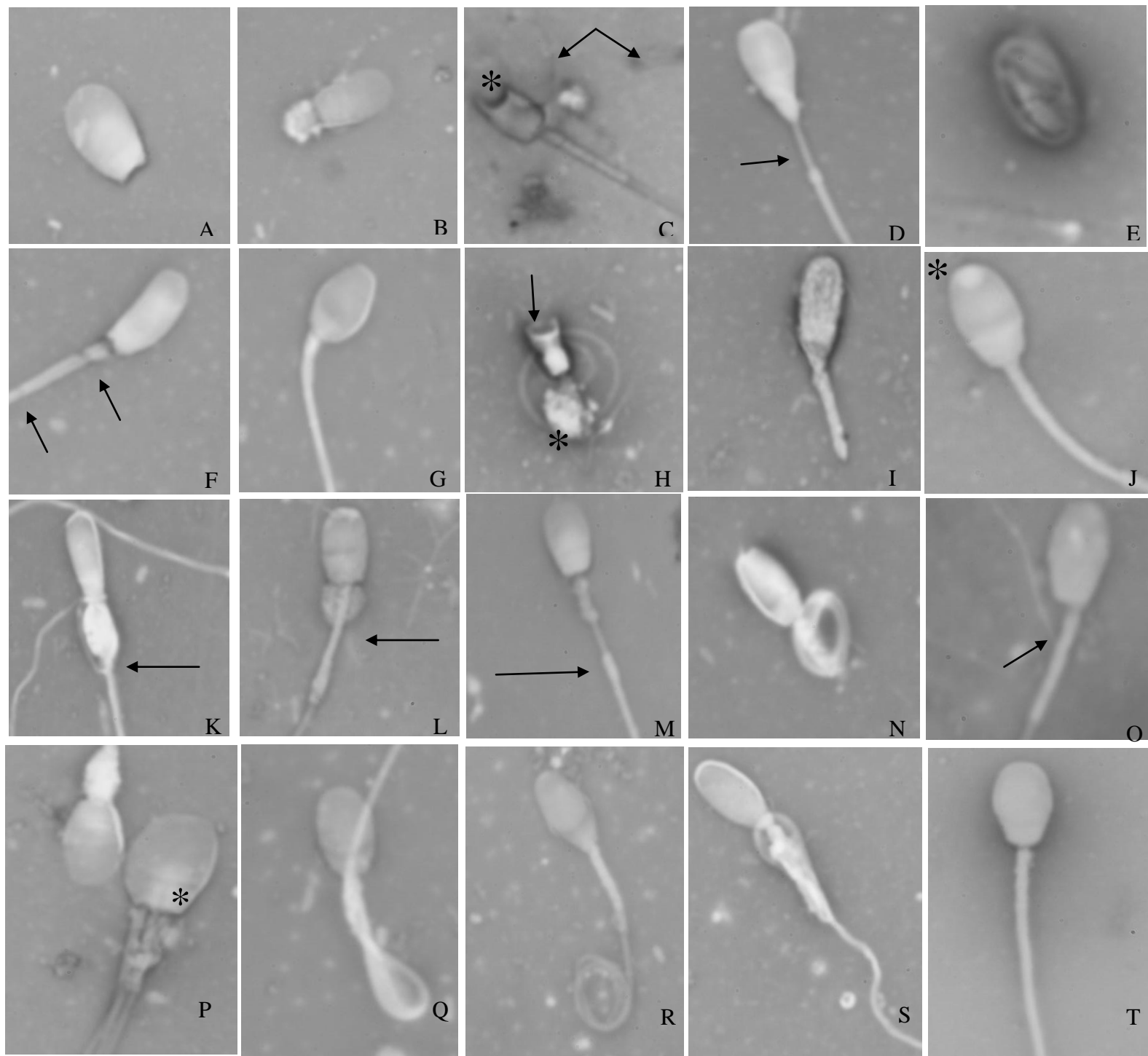


Figure 4. 2. The morphology of different sperm abnormalities found in alpaca ejaculates taken 1000x under oil immersion. A. Normal alpaca head with missing mid and principle pieces. B) Stump tail with a proximal droplet. C) *Large vacuole found in the apical region of the head. Arrows depict decapitated sperm with detached midpiece from the capitulum. D) Pyriform head shape of sperm with absent midpiece (arrow). E) Teratoid sperm. F) Tapered head shape abnormality with segmentations in the midpiece (arrows). G) Pear-shaped head abnormality H) Microcephalic abnormality with a large apical vacuole (arrow) and coiled principle piece. '*' Depicts proximal droplet. I) Tapered head with a short tail defect (missing principle piece) and heterogeneous chromatin appearance in the head. J) Beaded acrosome defect. K) Tapered head; Arrow is pointing to the proximal droplet L) Normal head sperm morphology with a prominent proximal droplet (arrow). M) Short or interrupted midpiece (arrow). N) Coiled mid and principle pieces. O) Abaxial tail. P) *, Macrocephalic sperm with a shattered midpiece and accessory sperm tails. Q) Distal midpiece reflex with intact midpiece and normal head morphology. R) Coiled principle piece and abnormal head shape S) Distal midpiece reflex with shattered midpiece. T) Normal sperm morphology.

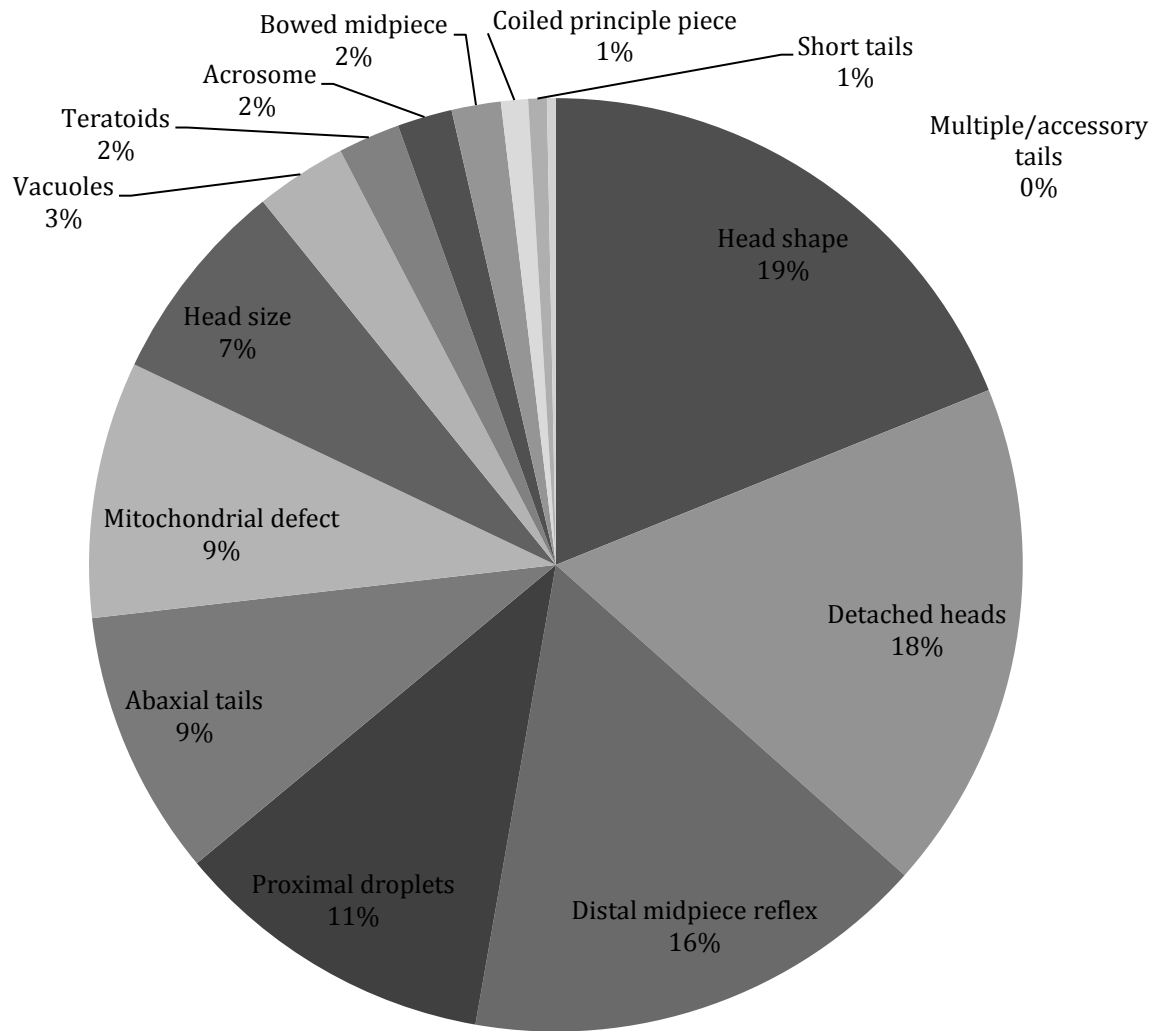


Figure 4. 3. Overview of alpaca sperm morphology abnormalities. Values represent the percentage of each particular defect among all defects found in 199 ejaculates collected from 47 different males.

Table 4. 1. Morphometry of accessory sex glands and plasma testosterone concentration in alpacas (n=47).

	Age (years)	Body weight (kg)	Plasma testosterone (ng/mL)	Testes area (left & right combined) (cm ²)*	Prostate area (left & right combined) (cm ²)**	Bulbourethral area - left & right combined (cm ²)**
Mean	7.3±0.4	56.2±1.0	4.8±0.5	14.1±0.4	45.9±2.3	31.1±1.0
±SEM						
Range	4-14	43-74	0.9-15.5	8.8-20.7	16.8-82.6	17.5-53.1

* Measurement done by vernier calipers

** Measurement done by transrectal ultrasonography

Table 4. 2. Physical and chemical characteristics of ejaculates collected from alpacas by artificial vagina (n=243 ejaculates).

	Duration (min)	Volume (semen*; mL)	Volume (froth**; mL)	Viscosity (cm)	pH	Sperm concentration (x 10 ⁶ /mL)	Sperm motility
Mean ± SEM	23.6±0.5	1.7±0.1	5.09±0.3	3.1±0.2	7.6±0.1	77.3 ± 9.1	39.6± 1.9
Range	5-30	0-12	0-20	0-18	6.4-8.8	0-1,373	0-100

* Semen volume is the measurement of the liquid portion of the ejaculate

** Froth volume is the measurement of the foam portion of the ejaculate

Table 4. 3. Morphologic characteristics of sperm collected from adult alpacas by artificial vagina (mean \pm SEM; n= 199 ejaculates).

Mean	53.8 \pm 1.6	37.2 \pm 1.3	37.6 \pm 1.3	20.5 \pm 1.1	9.7 \pm 0.84	8.6 \pm 0.61	1.3 \pm 0.28	1.3 \pm 0.21
(%)								
Range	0-98	0-90	1-100	0-91	0-80	0-45	0-30	0-25
(%)								

Table 4. 4. Distribution of total OIF/NGF abundance in alpaca seminal plasma collected artificial vagina (n=153).

Quantity of OIF/NGF per ejaculate	Proportion by ejaculates (%)	Ovulation rate observed in Tanco et al., 2011[49]
$\leq 60 \mu\text{g}$	4/153 (2.6%)	--
$60 < \text{OIF/NGF} \leq 125 \mu\text{g}$	6/153 (3.9%)	30
$125 < \text{OIF/NGF} \leq 250 \mu\text{g}$	5/153 (3.3%)	70
$\geq 250 \mu\text{g}$	138/153 (90.2%)	90

Table 4. 5. Correlation (Spearman Rho coefficient; $P < 0.05$) among male reproductive parameters: A = age of male; B = weight of male; C = duration of breeding; D = OIF/NGF concentration; E = Total OIF/NGF per ejaculate; F = sperm concentration; G = pH; H = volume of semen; I = sperm motility; J = semen viscosity; K = plasma testosterone concentration; L = total testis area; M = total prostate area; N = total bulbourethral area; O = % live sperm per ejaculate; P = % normal sperm per ejaculate.

	A	B	D	E	F	G	H	I	L	N	P
A					-0.19*						
B					0.33						
C				0.36					0.25	0.28	
D					0.27	-0.24					
E			0.70								
F	-0.19*	0.33	0.27			-0.41					
G			-0.24		-0.41		0.32	-0.33			
H			-0.19*	0.49		0.32					
I		0.28			0.37	-0.33					
J		-0.35	-0.26		-0.19*		0.23				
K											
L											
M	0.37	0.24	0.21*	0.22							0.19*
N	0.38	0.20*	-0.19*								
O	-0.20*				0.47	-0.33		0.76	0.29		0.24
P		0.22			0.34	-0.26*					

* Tendency for a correlation (P<0.10)

Table 4. 6. Stepwise multiple regression analysis results: Dependent variable: Total OIF/NGF per ejaculate (n=153)

Independent variables	β - weight	t value	P-value
OIF/NGF Concentration	0.63	8.11	<0.0001
Semen volume	0.424	5.54	<0.0001
Prostate area	0.22	2.84	0.006
Constant		-3.25	0.002
model R= 0.75; R^2 =0.56; Adjusted R^2 =0.54; F= 33.22 ; p<0.0001			

4.5 Discussion

The abundance of OIF/NGF in alpaca semen and its relationship with reproductive parameters are reported in these studies. OIF/NGF constituted approximately 45.2% of total seminal plasma protein in alpaca. However, high individual variability existed not only in seminal plasma OIF/NGF concentration, but also with total protein. Azoospermic samples were found in only 9.1% of samples which corresponds with the range that has been reported previously (5% to 22% [71, 84, 298, 299]).

The dose of OIF/NGF is important in triggering LH secretion and inducing ovulation [49, 52]. A 60 µg dose administered intramuscularly corresponded with a 30% ovulation rate; increasing the dose to 250 µg induced ovulation in 90% of llamas treated[49].

Only, 2.6% of ejaculates contained less than 60 µg of OIF/NGF. We hypothesize that these ejaculates do not contain sufficient OIF/NGF to induce ovulation during copulation which may contribute to ovulation failure in camelids. It is noteworthy that the 90% ovulation rate found in llamas and alpacas directly corresponds to the percentage of ejaculates containing 250 µg or greater. This suggests that the 10% ovulation failure rate could also be attributed to low OIF/NGF in the ejaculate as well as female-derived pathologies.

Interestingly, a relationship between OIF/NGF and sperm was observed. Sperm concentration was correlated with OIF/NGF concentration but not with total OIF/NGF per ejaculate. It is unclear whether OIF/NGF is present in alpaca sperm as two immunohistochemical studies using different antibodies came to different conclusions [250, 300]. However, NGF has been characterised in bovine sperm[249]. In the present study, sperm cells were separated from

seminal plasma by low centrifugation to ensure sperm membranes remained intact. Thus, it is unlikely that sperm-derived proteins contributed to the OIF/NGF quantified in seminal plasma.

Unlike in the golden hamster [248] or men [255], a linear relationship between OIF/NGF and sperm motility was not observed. However, this may be the consequence of collection method and the difficulty of assessing progressive motility in camelid semen due to viscosity.

The primary source of OIF/NGF in camelids is the prostate gland [300]. Correspondingly, a significant relationship between total OIF/NGF per ejaculate and male prostate size was observed during this study. The camelid corpus of prostate has two lateral (right and left) entities that are connected by a smaller isthmus. A possible reason as to why a stronger correlation was not observed between OIF/NGF abundance and prostate area may be because the measurements of the largest diameter of the right and left prostate lobes were used to estimate total prostate size. In reality, the area of the prostate is significantly larger when the whole organ is measured. In addition, the measurement of the *corpus* does not include the amount of OIF/NGF-containing secretory units found in the *disseminate* prostate [301]. In agreement with histology data, testis and bulbourethral gland had no influence on total OIF/NGF content.

The mean ejaculate volume observed was 1.7 ± 0.12 mL and the estimated froth volume was 5.1 ± 0.27 mL. The estimated froth/foam volume did not take into consideration the density or compaction of bubbles. The presence of froth/foam is a consequence of using artificial vagina as a collection method. In one study, approximately 11% of ejaculates contained $\geq 40\%$ of foam when artificial vagina collection method was used [71]. Volume measurements in the present study did not take foam/froth volume into consideration. However, it is most likely that during copulation, foam/froth volume is liquefied *in vivo* and could potentially increase total semen

volume and total OIF/NGF abundance. Thus, it is a plausible explanation as to why correlations between total NGF in the ejaculate and sperm parameters such as motility and concentration were not observed.

We conclude that the amount of OIF/NGF in camelid semen is variable within and among males and may be related with sperm parameters such as concentration, but has no influence on sperm motility, viability or morphology. Our future objective is to determine if the quantity of OIF/NGF in semen is associated with ovulation and pregnancy rates in alpacas.

4.6 Acknowledgements

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5.0 RELATIONSHIP BETWEEN OVULATION-INDUCING FACTOR/NERVE GROWTH FACTOR (OIF/NGF) IN SEMINAL PLASMA AND FERTILITY IN ALPACAS

5.1 Abstract

Two experiments were conducted to determine whether factors associated with mating correlated with fertility in alpacas. We hypothesized that levels of OIF/NGF in semen would be one of the candidates that could be used as an indicator of male fertility in terms of ovulation, pregnancy and live birth rates. For both experiments, semen was collected (n=3 to 6 ejaculates/male) using an artificial vagina inserted into a phantom mount. Semen volume, pH, sperm concentration, motility, viability and morphology were recorded for each sample. The concentration of OIF/NGF in seminal plasma was determined by radioimmunoassay. In Experiment 1, the breeding history of twenty-two males from the previous year was taken as their fertility assessment; i.e., the number of live births from the number of females that were bred. In Experiment 2, males were selected at random from a larger herd to determine if the amount of OIF/NGF in semen could discriminate between more and less fertile males. Data from males were combined based on total OIF/NGF abundance and expressed as low (n=2 males; 0.8 ± 0.4 mg/mL), medium (n=4 males; 3.7 ± 0.7 mg/mL) and high (16.2 ± 5.7 mg/mL; n=2 males) of OIF/NGF. A total of 160 controlled breedings (n=20 females/ males) were conducted. Females were bred once when a growing ovarian follicle ≥ 7 mm was detected by daily ultrasonography. Ovulation was assessed on Day 3 (Day 0= day of breeding) and confirmed on Day 7 by the presence or absence of a CL. Ultrasonography was used to measure the diameter of the CL on Days 7 and 20, and diagnose and confirm pregnancy on Days 20 and 25, respectively.

In Experiment 1, the number of live births tended to be related to OIF/NGF concentration ($\rho = 0.38$; $P = 0.09$) and total OIF/NGF per ejaculate ($\rho = 0.36$; $P = 0.09$). In Experiment 2, ovulation rates were not different among the low, medium or high OIF/NGF groups (32/40 [80%], 62/80 [78%] and 36/40 [90%], respectively, $P = 0.13$). Similarly, pregnancy rates were not different among groups (18/40 [45%], 41/80 [51%], 16/40 [40%], respectively, $P = 0.47$). Pregnancy rates were strongly correlated with sperm concentration ($\rho = 0.65$; $P = 0.04$) and viability ($\rho = 0.85$; $P = 0.004$), and tended to be correlated with sperm motility ($\rho = 0.55$; $P = 0.08$) and morphology ($\rho = 0.49$; $P = 0.10$). Retrospective comparison of males of high and low fertility suggested a relationship between fertility and the concentration of OIF/NGF in seminal plasma; however, results of a prospective comparison of males with low, medium and high concentrations of OIF/NGF did not support the hypothesis that seminal OIF/NGF can be used as a predictor of fertility in alpacas.

5.2 Introduction

South American camelids are induced ovulators [185]. As such, copulation is required not only for the delivery of sperm for fertilization, but also for the release of the oocyte from the ovarian follicle [302]. Coitus in camelids is associated with an increase in the circulating concentration of luteinizing hormone (LH) that precedes ovulation [151, 160, 303]. The LH secretion profile observed in females after copulation (with an intact or vasectomized male [151, 160, 186]) also occurs after intramuscular administration of homologous seminal plasma [42, 45, 48, 54]. Hence, it is unlikely that penile stimulation of somatosensory nerves is the primary stimulus for ovulation in these animals. Furthermore, ovulation occurred in alpacas after intrauterine deposition of seminal plasma with endometrial abrasion, but not occur after intrauterine deposition of saline with or without abrasion [13, 47]. The ovulation-inducing factor (OIF) responsible has been identified as the well-known neurotrophin, β -nerve growth factor (β NGF) [39-41]. The potency of this factor has been demonstrated in several studies [39, 40, 49, 292] where more than 90% of female llamas or alpacas, isolated from male contact, ovulated after a single dose of seminal plasma or purified OIF/NGF. A dose representing $1/200^{\text{th}}$ ($60 \mu\text{g}$) of OIF/NGF found in a normal ejaculate caused a 30% ovulation rate, and increasing the dose to $1/50^{\text{th}}$ ($250 \mu\text{g}$) resulted in ovulation rates of $\geq 90\%$ [49]. In a recent study, as little as $10 \mu\text{g}$ of human recombinant NGF induced ovulation in 60% (3/5) of alpacas while all animals (5/5) ovulated when the dose increased to $100 \mu\text{g}$ or $1000 \mu\text{g}$ [254]. This dose-related response appears to be related to the amount of LH that is released by the adenohypophysis [49] [47]. In two independent *in vitro* studies, alpaca seminal plasma or OIF/NGF purified from llama seminal plasma stimulated LH release from gonadotrope cells in culture, and the concentration of LH secretion was related to the dose [52, 53]. These results correspond with those of *in vivo* studies

[42, 45, 54] and may be the basis for a luteotrophic effect (greater CL diameter and progesterone concentration) observed with increasing doses of OIF/NGF [47, 49].

We hypothesize that the total amount of OIF/NGF per ejaculate is related to male fertility and can be used to discriminate between more and less fertile males. Rationale for the hypothesis is based on: 1) OIF/NGF elicits its response in a dose-dependent manner in llamas and alpacas, 2) OIF/NGF is luteogenic, 3) the amount of OIF/NGF is variable among male camelids (Thesis Chapter 4), and 4) the concentration of OIF/NGF tends to be higher in semen samples with greater sperm concentration (Thesis Chapter 4). The present study was designed to determine if the abundance of OIF/NGF in alpaca semen is associated with a) previous fertility among males (retrospective comparison, Experiment 1) and b) male-induced ovulation and pregnancy rates (prospective comparison, Experiment 2).

5.3 Materials and methods

Data collection for live-animal endpoints were conducted during January to April at the Quimsachata Research Station in the Department of Puno, Peru (15°S, 71°W, and 4500 m above sea level). Laboratory analyses were conducted at the University of Saskatchewan, Canada. Experimental procedures were performed in accordance with the animal care protocols established by the University of Saskatchewan.

5.3.1 Experiment 1- Retrospective study on semen OIF/NGF concentrations and live birth rates

Semen was collected by artificial vagina [84] from 22 male alpacas that had been used during the previous breeding season and for which pregnancy rates for that season were known. The males varied in age (from 4-14 years), weight (from 44-74 kg), breed (Huacaya or Suri). Ejaculates were collected twice on a given day, at an interval of ≥ 5 hr. Males were given at least one full day of rest between collection days (n=3-6 ejaculates per male). After collection, an aliquot of semen was taken to assess sperm concentration, motility, viability and morphology. Ejaculates were centrifuged at 500 x g for 15 minutes to remove spermatozoa without disrupting the sperm cell membrane. The supernatant was collected and centrifuged for 1500 x g for 15 minutes to ensure only seminal plasma remained. Samples were stored in liquid nitrogen for the duration of the study in Peru, and then stored at -80°C in Canada until OIF/NGF assay.

The overall mean OIF/NGF concentration for a given male was the average of the concentrations measured in individual ejaculates for that male. The mean concentration for each male was used to correlate the number of offspring produced relative to the number of females bred during the previous breeding season. The 22 males were involved in a total of 171 breedings. Nonpregnancy was diagnosed (or assumed) if the female was re-bred during the same breeding season or failed to give birth, and pregnancy was confirmed by live birth during the following birthing season. Breedings were controlled and recorded by staff from the Quimsachata Research Station. Birth rates were calculated as the number of live offspring relative to the number of breedings by a given male.

5.3.2 OIF/NGF assay

A double-antibody radioimmunoassay for OIF/NGF, previously validated for use with seminal plasma (Thesis Chapter 3), was used. The primary antibody was a rabbit antihuman-OIF/NGF polyclonal antibody (Santa Cruz Biotechnology, Dallas Texas, USA). The secondary antibody was goat serum raised against rabbit immunoglobulins [271]. Samples were assayed in duplicate with a standard curve ranging from 0 to 200 $\mu\text{g/mL}$. The minimum detectable limit was 10 $\mu\text{g/mL}$. All seminal plasma samples were measured within the same assay to remove interassay variation. The intra-assay coefficients of variation for low (25 μg) and high (100 μg) references were 10% and 6%, respectively. Ejaculates were diluted with PBS to fit within the standard curve. Values were then corrected by the dilution factor to calculate the concentration found within seminal plasma. Total OIF/NGF abundance within an ejaculate is calculated by multiplying the concentration of OIF/NGF measured by the total volume of semen collected.

5.3.3 Experiment 2 – Prospective breeding trial

Adult male alpacas ($n=8$) were selected at random from a herd of 47 males. Semen samples (3 to 6 ejaculates/male) were collected and processed as described for Experiment 1, and OIF/NGF concentrations in seminal plasma were assayed. Based on the mean concentration OIF/NGF, males were categorized into low ($n=2$ males; $0.8 \pm 0.4 \text{ mg/mL}$), medium ($n=4$ males; $3.7 \pm 0.7 \text{ mg/mL}$) and high ($16.2 \pm 5.7 \text{ mg/mL}$; $n=2$ males) OIF/NGF groups, and pregnancy rates were compared among male groups in a subsequent breeding trial.

Mature, non-pregnant, non-lactating, female alpacas ($n=160$) ranging from 3 to 12 years were used. The alpacas were selected from a group of 200 based on an initial examination to confirm that they were in adequate body condition and were non-pregnant with no apparent

pathological conditions of the reproductive tract, as detected by transrectal ultrasonography (7.5 MHz linear array transducer, MyLab Five, Esaote, Italy). Ovarian follicle development was examined once daily [304] for at least three consecutive days by transrectal ultrasonography before breeding. Alpacas with a growing follicle ≥ 7 mm in diameter in either ovary were assigned randomly to one of the eight males for breeding. Breedings were done in the same location as semen collection to minimize potential effects of environment on male behaviour, and males were allowed to breed the female for a maximum of 30 minutes. The breeding (pair) was monitored during copulation to ensure proper vaginal intromission had occurred. If copulation was interrupted at <30 minutes, the male was given the opportunity to mount the same female again; if he showed no interest, the male was removed and the total intromission time was recorded. Two females were bred to an individual male per day, one in the morning and one in the afternoon. At least one full day of rest was given to each individual male before the next day of mating. A total of 160 copulations were monitored (20 females/ male x 8 males).

Females were examined by transrectal ultrasonography on Day 3 (Day 0 = day of copulation) to detect ovulation. Ovulation was defined as the absence of a pre-ovulatory sized follicle (≥ 7 mm) which had been present at the time of breeding, and confirmed by detection of a CL on Day 7 [48]. Transrectal ultrasonography was done on Days 7, 20 and 25 to determine CL diameter and pregnancy status. A definitive diagnosis of pregnancy was based on detection of an embryo proper with a heart beat [305]. Pregnant females were examined again on Days 30 and 40 to assess maintenance of pregnancy. Pregnancy rate was defined as the number of females that were diagnosed pregnant from the number of females that were bred.

5.3.4 Statistical analyses

Statistical analyses were done using the Statistical Analysis Software package (SAS Learning Edition, 9.1; SAS Institute Inc., Cary, NC, USA). *Experiment 1*: Associations between OIF/NGF concentration or total OIF/NGF abundance in semen with live birth rates were assessed using nonparametric Spearman rank correlation tests, given the non-normal distribution of these variables. *Experiments 2*: Data from males with similar abundance of OIF/NGF in their ejaculates were combined and categorized as low (n=2 males), medium (n=4) and high (n=2) OIF/NGF groups. Ovulation and pregnancy rates were compared among groups by Chi-Square analysis (proc genmod procedure, SAS). Non-serial data (i.e., follicle diameter on Day 0, CL diameter on Day 7, and maximum CL diameter) were compared among OIF/NGF groups by analysis of variance. All values are expressed mean \pm SEM.

5.4 Results

5.4.1 Experiment 1- Retrospective study on OIF/NGF concentration and live birth rates

According to the reproductive records provided by the research station, the number of females bred per male ranged from 3 to 13. The birth rates (number of live offspring from the number of breedings by a given male) obtained from the 22 males for the previous breeding season ranged from 14% to 100%. The number of live births per male tended to be positively correlated with OIF/NGF concentration ($\rho=0.38$; $P=0.09$) and total OIF/NGF per ejaculate ($\rho=0.36$; $P=0.09$).

5.4.2 Experiment 2 – Prospective breeding trial

The age of the females, copulation time, and follicle diameter at breeding was similar among low, medium and high OIF/NGF groups (Table 5.1). Copulation with males containing low, medium or high amounts of OIF/NGF in their ejaculate was not associated with ovulation or pregnancy rates (Table 5.1). Ovulation rates induced by individual males ranged from 40% to 100% and were not correlated with OIF/NGF concentration ($\rho=0.33$; $P=0.42$) or pregnancy rate ($\rho=0.05$; $P=0.45$; Table 5.2). Spearman's rank correlations on variables associated with pregnancy are shown in Table 2. Sperm concentration, viability and sperm morphology were all important factors related to pregnancy ($P\leq 0.05$) while sperm motility tended to be related with higher pregnancy rates. Higher pregnancy rates were correlated with younger female alpacas ($\rho=-0.78$; $P=0.01$).

Table 5. 1. Summary of the ovarian response (mean±SEM) in female alpacas (n=160) bred to males containing low (n=2), medium (n=4) and high (n=2) concentrations of OIF/NGF in their ejaculates (Experiment 2).

Male group	Low	Medium	High
Number of females	40	80	40
Age of females	6.6 ±0.39	6.5±0.41	6.9±0.42
Duration of mating (min)	22.1±1.08	21.9±0.98	23.1±1.20
Follicle diameter at mating (mm)	9.2±0.23	9.1±0.26	9.4±0.23
Ovulation rate	32/40 (80%)	62/80 (78%)	36/40 (90%)
Pregnancy rate	18/40 (45%)	41/80 (51%)	16/40 (40%)
CL diameter on Day 7* (mm)	13.0±0.30	12.9±0.25	12.9±0.28

*Day 0 = day of mating

Table 5. 2. Correlations between pregnancy rate and breeding parameters in alpacas (Experiment 2)

Factor	Spearman-rho correlation coefficient ρ	P-value
Total OIF	0.05	0.45
Sperm concentration	0.65	0.04 [*]
Sperm motility	0.55	0.08
Sperm viability	0.85	0.004 [*]
Semen pH	-0.76	0.01 [*]
Total testis area	-0.68	0.03 [*]
Normal morphology	0.49	0.10
Midpiece defect	-0.72	0.02 [*]
Proximal droplet	-0.60	0.06
Acrosome defect	-0.88	0.002 [*]
Age of female	-0.78	0.01
Maximum CL diameter	0.15	0.36

*Correlation is significant at the 0.05 level (2-tailed)

5.5 Discussion

The results of Experiment 1, a retrospective correlation of male fertility and semen OIF/NGF content, were consistent with the hypothesis that the abundance of OIF/NGF in semen is related to male fertility, but results of Experiment 2, a prospective study, did not support the hypothesis that the abundance of OIF/NGF in semen can be used to predict the degree of male fertility. The dichotomy in results of the two experiments justifies further investigation into the relationship between OIF/NGF in seminal plasma and male fertility. Limitations of Experiment 2 were the relatively small number of males used to make the correlations, and the potentially independent effects of sperm quality and OIF/NGF in seminal plasma. Ovulation rates induced by individual males ranged from 40% to 100%. The male with the most OIF/NGF in his ejaculate, also had the highest ovulation rate but one of the lowest pregnancy rates. Analysis of his semen revealed very low sperm concentration and numerous sperm defects. The ovulation rate of the male with the least OIF/NGF per ejaculate was among the lowest but sperm quality (normal morphology, motility and concentration) was high. The low OIF/NGF male had a greater percentage of normal sperm which lead to higher pregnancy rates (35% vs. 60% pregnant for the high OIF/NGF and low OIF/NGF male respectively).

Variation among ejaculates from the same male may also have contributed to the dichotomy in results of the two experiments. The amount of OIF/NGF ejaculated by each male was estimated by semen collection using an artificial vagina inserted into a phantom mount. However, the amount of OIF/NGF in semen is not equal and in a previous study great variation was found both within and among ejaculates of male alpacas (Bogle PhD thesis, Chapter 4). This variation was not found in all males: some males ejaculated relatively similar amounts of OIF/NGF, semen volume and sperm concentration while others were inconsistent. This may

have been one of the most important factors in limiting our ability to test the hypothesis stated in Experiment 1. The mean percentage of normal sperm (37%) observed in ejaculates was similar to that reported by Lichtenwalner et al. [84] but considerably lower to what was reported by Bravo et al. [306]. Low pregnancy rates could also be related with mounting efficiency/intra-anal intromission instead of intrauterine. All males were monitored for vaginal intromission at the beginning of breeding but frequent monitoring was not done. Once the male had dismounted, on several occasions, the female had anal swelling, an agape anus or semen around the perineum. A study designed to characterize the ejaculatory pattern in llamas reported 26 of 71 intromissions were in the anus and this phenomenon occurred in 3 of the 5 male llamas that were used [80].

Previous studies have demonstrated that administering seminal plasma or purified OIF/NGF from seminal plasma will trigger LH release, ovulation and CL development in a dose dependent manner [49, 52]. However, in the present study, the route of administration changed from intramuscular to intrauterine (via copulation) and included other male-derived factors associated with breeding. Evaluation of the ejaculates in the prospective experiment revealed two important factors that may have confounded the results: 1) the mean OIF/NGF content in the seminal plasma of the low group was ≥ 250 μg , and 2) many of the ejaculates collected had a significant volume of froth. The amount of 250 μg was the minimum dose of seminal plasma derived OIF/NGF reported to generate a 90% ovulation rate in llamas given intramuscularly [49], while the dose of 100 μg of human recombinant NGF induced ovulation in all alpacas [254]. Perhaps no significant differences were observed in the present study because OIF/NGF concentrations in even the low group exceeded that necessary to induce ovulation. The presence of froth is commonly observed in ejaculates collected from llamas and alpacas using an artificial vagina. Quantification of total OIF/NGF in the ejaculate was expressed as the product of semen

volume and OIF/NGF concentration; however, the volume of foam was excluded because of the inability to consistently measure its volume. Hence, the total volume and amount of OIF/NGF deposited into the uterus during normal copulation was underestimated in the measurements used in the present study. To emphasize the potential impact this may have had, intrauterine deposition of 1 mL diluted seminal plasma (1:1) did not induce ovulation in alpacas [48] but ovulations (41%) occurred when the volume increased to 2 mL diluted seminal plasma (1:1) [13]. The quantity of OIF/NGF in the seminal plasma was not known in the latter studies, but the volume used for ovulation induction was less than the mean volume used in the present study (1.7 mL undiluted; Bogle thesis, Chapter 4). Further studies to investigate the absorption of OIF/NGF by the endometrium is needed. In addition, a dose-response study should be conducted to determine if the intrauterine deposition of OIF/NGF can induce ovulation.

The effect of mating frequency on ovulation and pregnancy rates have been reported in alpacas. Males bred twice for nine consecutive days had a 81% ovulation rate and 78% pregnancy rate [307]. Although ovulation rates were not different when breeding frequency was increased to four and six times a day, a decline in the number of pregnant animals was observed with six breedings [307]. In this study, males were: 1) allowed to breed only two females per day; 2) breedings were separated by a minimum of four hours; 3) breeding days were not consecutive but were separated by one full day. Consequently, duration of breeding, pregnancy and ovulation rates were not influenced by the schedule or day of breeding. Of the 160 pairings, there was only one (0.6%) isolated event when a male showed no libido and did not mate the female which may suggest physical exhaustion or lack of sexual interest.

The fertilization rate of alpaca embryos collected from sacrificed animals three days post mating was 88% [308]. Based on this observation, we assume that there was a total fertilization

rate of 70% (128 (total number of ovulations) x 0.88 (fertilization rate)/ 160 (total number of breedings)). A pregnancy rate of 47% indicates an embryonic loss of 23% which is low in comparison to other studies. A loss of approximately 50% embryos within the first month of gestation has been reported previously in alpacas [308].

In conclusion, retrospective correlation of male fertility and semen OIF/NGF content was consistent with the hypothesis that the abundance of OIF/NGF in semen is related to male fertility, but the hypothesis was not supported by results of a prospective test. Further testing is warranted in studies that better control for variation among ejaculates within males, and provide more distinct selection of males with low vs. high OIF/NGF content in their semen.

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6.0 POWER FLOW DOPPLER ULTRASONOGRAPHY TO ASSESS A LUTEOTROPHIC EFFECT OF OIF/NGF AFTER MATING IN PREGNANT AND NONPREGNANT ALPACAS

6.1 Abstract

Ovulation-inducing factor/nerve growth factor (OIF/NGF) is a seminal plasma protein that induces ovulation and CL formation in camelids in a dose-dependent manner. A luteotrophic effect of OIF/NGF has been reported in llamas, alpacas, and cattle. To test the hypothesis that CL form and function is enhanced in female alpacas mated to males with more OIF/NGF in their ejaculate, we compared changes in CL vasculature by power flow Doppler ultrasonography and circulating concentrations of progesterone in pregnant and nonpregnant alpacas bred to males with varying quantities of OIF/NGF in their ejaculates. Female alpacas with a growing follicle of 7 -12 mm were bred to a male with low (n=2 males; 0.8 ± 0.4 mg/mL; n=8 females), medium (n=4 males; 3.7 ± 0.7 mg/mL; n=16 females) or high (n=2 males; 16.2 ± 5.7 mg/mL; n=8 females) concentrations of OIF/NGF in their ejaculate. Cineloops of ultrasound images of the ovary containing the developing CL were recorded in brightness- and power flow Doppler-modalities. Alpacas were examined every 4 hours from the time of breeding (Day 0) until ovulation, and every other day until Day 20. If pregnant, alpacas were scanned again on Days 30 and 40. Doppler recordings of the ovary containing the CL were taken from the caudal to cranial pole. Still-frame images corresponding to cross-sections at 1/4, 1/2 (maximum CL diameter) and 3/4 of the way through the CL were used for image analysis to estimate vascular area. Positive correlations were found between CL diameter and plasma progesterone concentration ($r=0.68$; $P<0.0001$), CL diameter and vascular area ($r=0.53$; $P<0.0001$), and progesterone concentration and vascular area ($r=0.35$; $P<0.0001$). The male groups (low-, medium-, and high-OIF/NGF in

ejaculates) did not influence LH secretion, CL diameter, progesterone concentration or CL vascularization in pregnant and nonpregnant alpacas. The first significant reduction in progesterone concentration ($P<0.01$) and blood flow area ($P=0.05$) occurred on Day 9 in nonpregnant alpacas whereas a decrease in CL diameter using B- mode was not detected until Day 11 ($P\leq 0.01$). Results did not support the hypothesis that CL form and function is enhanced in female alpacas mated to males with more OIF/NGF in their ejaculate. Differences in CL blood flow area, detected by power flow Doppler, were apparent in pregnant vs. non-pregnant alpacas by 9 days after mating.

6.2 Introduction

The corpus luteum (CL) is an endocrine gland that develops after rupture of the dominant follicle during ovulation [161, 309]. The dynamic process of CL formation involves rapid tissue remodeling, angiogenesis, cell differentiation, synthesis of various proteins and steroids, followed by rapid regression in non-pregnant animals [310-314]. Maintenance of the CL and elevated concentrations of progesterone are essential for sustaining pregnancy in alpacas; i.e., removal of the CL anytime during the first 9 months of gestation resulted in abortion within two days [315]. Circulating concentrations of progesterone remain elevated throughout pregnancy, until a steady decline beginning 72h before parturition [316]. In addition, rescue of the CL from luteolysis between 8 and 10 days after ovulation has been implicated in maternal recognition of pregnancy in llamas [186], and higher plasma progesterone concentrations during the first week of gestation have been shown to improve embryo survival in cattle [317]. Since CL development is vital in the establishment and maintenance of pregnancy, luteotrophic factors may improve reproductive efficiency by enhancing CL function.

Nerve growth factor (NGF) is a protein that exerts a physiological response in several biological processes including nervous tissue development [35, 36, 318], angiogenesis [319-322], male reproduction [46, 248, 249, 255, 323], ovarian follicular development [227, 231, 232, 324, 325] and ovulation-induction [39, 40]. Recently, it has been reported that the seminal plasma protein described as an ovulation-inducing factor (OIF) in llamas and alpacas is β -NGF, based on similar molecular, structural and functional properties [39, 40]. For the purpose of this report, OIF/NGF will be used to denote β -NGF of seminal plasma origin.

Several methods may be used to induce ovulation in camelids, such as mating, the administration of seminal plasma, or treatment with hormones such as OIF/NGF, GnRH or LH

[38, 130, 159, 185]. In each method of induction, the ovulatory mechanism results in an increase in circulating concentrations of LH [159, 160, 326]. A luteotrophic effect of seminal plasma or OIF/NGF has been reported in several studies in which treatment resulted in a longer CL lifespan or higher concentrations of progesterone in circulation in comparison to animals given GnRH [48, 49, 55, 175, 293]. In addition, *in vitro* perfusion of mid-luteal stage CL with NGF stimulated progesterone secretion [234]. Furthermore, LH secretion *in vivo* and *in vitro* was higher with increasing doses of OIF/NGF or seminal plasma, and CL function was directly related to the dose of OIF/NGF administered intramuscularly in camelids [45, 49, 53]. The mechanistic relationship between OIF/NGF and GnRH is unclear. Both hormones initiate the ovulatory mechanism through LH secretion in camelids [49, 172], but it appears that OIF/NGF acts indirectly through GnRH *in vivo* since pre-treatment with a GnRH antagonists before OIF/NGF treatment blocked the LH surge and ovulation [171].

The concept that OIF/NGF is a luteotrophic factor stemmed from studies in which plasma progesterone concentration was higher in llamas given OIF/NGF than GnRH even though the CL was of similar size [175, 240]. Llamas given two doses of OIF/NGF, one to induce ovulation and another at the time of ovulation-detection, had a longer CL lifespan, greater maximum CL diameter, and higher progesterone concentration than those given a single pre-ovulatory dose or a third dose 24 h after ovulation detection [51]. The LH secretion profile was associated with the luteotrophic response; i.e., LH secretion was more prolonged after treatment with OIF/NGF than with GnRH [48, 175, 180, 327]. In this regard, a correlation between the duration of the preovulatory LH surge and the degree of luteogenesis has been reported for other mammalian species [328, 329]. Furthermore, intrauterine infusion of seminal plasma in pigs was associated with larger corpora lutea and higher circulating concentrations of progesterone [57], and

intramuscular administration of seminal plasma or OIF/NGF in cows and pubertal heifers resulted in a more rapid increase in circulating progesterone concentration and delayed regression of the extant corpus luteum [55, 293]. The mechanism by which OIF/NGF influences luteal function is unclear, but may be mediated via an angiogenic stimulus. Vascular endothelial growth factor (VEGF) was found to be essential for corpus luteum angiogenesis and progesterone secretion [330], and NGF was found to stimulate VEGF expression in cultured neonatal rat ovaries [331].

The present study was designed to test the hypothesis that CL function is enhanced in female alpacas bred to males containing a greater quantity of OIF/NGF in their ejaculate. We determined 1) the relationships among CL diameter, vascular perfusion, and plasma progesterone concentration, 2) if the amount of OIF/NGF in semen influences CL blood flow, and 3) the difference in CL blood flow between pregnant and nonpregnant alpacas using power flow Doppler imaging.

6.3 Materials and methods

Alpacas at the Quimsachata Research Station in the department of Puno, Peru (15°S, 71°W, and 4500 m above sea level) were used during the rainy season (March and April). Hormone and image analyses were conducted at the University of Saskatchewan, Saskatoon, SK, Canada. Experimental procedures were performed in accordance with the animal care protocols established by the University of Saskatchewan.

6.3.1 Semen collection and OIF/NGF assay

Mature alpaca males (n=8) were selected at random from a herd of 47 males of proven fertility. The males were trained to serve an artificial vagina inserted into a phantom mount [84] for a three-week period before sample collections. Acceptance of the phantom mount was

encouraged by applying freshly collected vaginal secretions or urine from receptive females to the phantom mount and orifice of the artificial vagina. In some instances, a live receptive female was placed beside the phantom mount to train the male to associate it with copulation. Once the association was established, a live female was no longer used. After the 3-week training period, the males were allowed six independent opportunities to serve the phantom mount. Semen samples (3 to 6 ejaculates/male) were collected, centrifuged once at 500 x g to remove intact spermatozoa and the supernatant was centrifuged again at 1500 x g to remove any remaining cellular debris. An aliquot was visualized under a light microscope to ensure no sperm cells were present. Seminal plasma was stored in liquid nitrogen until radio-immunoassay.

The concentration of OIF/NGF was measured by validated double-antibody radioimmunoassay (Bogle et al., 2015, Chapter 3 PhD thesis). Purified, biologically active OIF/NGF isolated from pooled llama/alpaca seminal plasma was labeled with ^{125}I by the chloramine-T method [270]. The primary antibody was a rabbit anti-human NGF polyclonal antibody (Santa Cruz Biotechnology, Dallas Texas, USA) and was used at a dilution of 1:1000. The secondary antibody was goat serum raised against rabbit globulins (Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada [271]). Seminal plasma samples were diluted with PBS to a concentration that was within the range of assay detection. Values were then corrected for the dilution factor. Samples were assayed in duplicate with a standard curve ranging from 0 to 200 $\mu\text{g/mL}$. The lowest detectable limit of the assay was 10 μg . All seminal plasma samples were measured in a single assay. The intra-assay coefficient of variation for the low (25 μg) and high (100 μg) references were 10% and 6%, respectively.

6.3.2 Breedings, examinations and image capture

Mature, non-pregnant, non-lactating, female alpacas (n=32), ranging from 3 to 12 years of age, were selected randomly from a group of 160. Ovarian follicle development was examined by transrectal ultrasonography once daily [304] for at least three consecutive days before breeding. Females were assigned randomly to a male and allowed a single breeding when a growing follicle between 7 and 12 mm in diameter was detected (n=4 females per male). Breedings were done in the same location as semen collection to minimize potential effects of environment on male performance, and males were allowed to breed the female for a maximum of 30 minutes. The breeding pair was monitored during copulation to ensure proper vaginal intromission had occurred. Females were examined by transrectal ultrasonography every 4 h after copulation (Day 0 = breeding) until ovulation was detected or until 42h after treatment, and every-other-day until Day 20 to monitor CL development. A blood sample was taken before and at 1, 2, 3, 4 and 8 hours after copulation to determine changes in plasma LH concentration, and every-other-day until Day 20 to determine plasma progesterone concentration. If pregnant, alpacas were examined again on Days 30 and 40. Blood samples were collected into heparinized tubes by jugular venipuncture (BD Biosciences, Mississauga, Ontario, Canada). Samples were centrifuged at 1500 x g for 15 minutes and the cellular portion was decanted and the supernatant was stored frozen at -20°C.

For all examinations, three-second cine-loops of the ovary containing the CL were captured in both B-mode and power flow Doppler mode using a linear-array transducer with a frequency of 5 to 7.5 MHz (MyLab Five, Esaote North America, Inc., Indianapolis, IN, USA). Still-frame images corresponding to cross-sections at 1/4, 1/2 (maximum CL diameter) and 3/4 of the way through the cine-loop of the CL were used for image analysis to estimate vascular

area. This was done by calculating the total number of serial frames occupied by the CL and selecting frames at the 25th, 50th, and 75th percentile of those representing the CL. The percentage of blood-flow area was calculated as the mean number of colored (power flow) pixels tabulated from the three images of the CL divided by the total number of pixels (B-mode) of the CL, as described previously [313]. Gray-scale images were used to estimate the border of the CL and the inclusion limit of the pixels for analysis.

6.3.3 LH and progesterone assays

Plasma LH was measured using a double-antibody radioimmunoassay [332]. Samples were analyzed in duplicate in a single assay. The minimum detectable limit was 0.1 ng NIAMDDK-oLH-24. The range of the standard curve was 0.06 ng (80% ligand-labeled LH) to 8.0 ng (20% ligand-labeled LH). The intra-assay coefficients of variation for the low (0.45 ng) and high (2.2 ng) reference standards were 6% and 9%, respectively.

Plasma progesterone concentrations were measured using a commercially available double-antibody radioimmunoassay kit (Coat-A-Count Total Progesterone; Diagnostic Products Corporation, Los Angeles, CA, USA). Samples were analyzed in three assays with an inter-assay coefficient of variation of 4%, 2% and 6% for reference concentrations 1.9, 3.6 and 16.6 ng respectively.

6.3.5 Statistical analyses

Data from males with similar abundance of OIF/NGF in their ejaculates were combined and categorized as low (n=2 males; 0.8 ± 0.4 mg/mL), medium (n=4 males; 3.7 ± 0.7 mg/mL)

and high (16.2 ± 5.7 mg/mL; n=2 males) groups. Ovulation and pregnancy rates were compared among groups by proc Genmod in SAS (SAS Learning Edition, 9.1; SAS Institute Inc., Cary, NC, USA). Non-serial data (i.e., follicle diameter on the day of breeding and interval to ovulation) were compared among OIF/NGF groups by analysis of variance. Serial data (i.e., hormone concentrations, CL diameter, CL vasculature) were compared by analysis of variance for repeated measures (Proc-mixed in SAS) to determine the effects of OIF/NGF in seminal plasma, and pregnancy status. Least significant difference (LSD) was used as a post-hoc test. All values are expressed mean \pm SEM.

6.4 Results

6.4.1 Effect of OIF/NGF concentration

Follicle diameter at the time of mating was similar among male groups, and no differences were detected among groups in the proportion of females that ovulated or became pregnant (Table 6.1). The interval from breeding to ovulation was longest to shortest in the low, medium and high OIF/NGF groups, respectively, but differences were not significant ($P=0.28$; Table 6.1). Data from three alpacas were excluded from hormone and CL analysis because of double ovulation and twin pregnancy (one in the high OIF/NGF group), a short luteal phase of 5 days (one in the medium OIF/NGF group), and illness (one in the high OIF/NGF group). Comparisons for remaining end points were based on observations from 29 females (n = 8, 15 and 6 in the low-, medium- and high-OIF/NGF male groups, respectively).

Plasma LH concentrations were elevated by 1 hour after breeding, peaked at 3 hours and had not yet returned to basal levels by 8 hours after breeding (Fig. 6.1). Although plasma LH

concentration in the high OIF/NGF was numerically higher over the first four hours post-breeding, differences among groups were not significant.

B-mode and power Doppler images of the corpus luteum are illustrated in Figures 6.2 and 6.3. During the first 7 days after breeding, CL diameter and blood flow area tended to be higher in the high OIF/NGF group than the other groups ($P \leq 0.09$; Fig. 6.4). A treatment-by-time interaction ($P < 0.02$) in CL blood flow area of non-pregnant alpacas was attributed to a greater rise from Days 3 to 7 in the high OIF/NGF group than in the other groups (Fig. 6.5). In pregnant alpacas, the luteal blood flow area increased over time ($P < 0.001$), but was not influenced by male group (Fig. 6.6).

A similar pattern was observed in plasma progesterone concentrations. Progesterone concentration was numerically but not statistically higher in the high OIF/NGF group during the first 7 days in the non-pregnant alpacas (Figs. 6.4 & 6.5). In pregnant alpacas, plasma progesterone concentrations increased over time ($P < 0.001$), but were not influenced by male group (Fig. 6.6).

A comparison of the temporal changes in CL diameter, plasma progesterone concentration and CL blood flow area in pregnant versus non-pregnant alpacas (irrespective of male OIF/NGF group) is shown in Fig. 6.7. Plasma progesterone concentration and CL blood flow area were higher in pregnant vs. non-pregnant alpacas by Day 9, and CL diameter was greater by Day 11. Plasma progesterone concentrations decreased to <1 ng/mL on Day 11 in non-pregnant animals. Correlations were found between CL diameter and progesterone concentration ($r=0.68$; $P < 0.0001$), CL diameter and vascular area ($r=0.53$; $P < 0.0001$), and progesterone concentration and vascular area ($r=0.35$; $P < 0.0001$).

Table 6. 1. Summary of the ovarian response (mean±SEM) in female alpacas bred to males containing low (n=2), medium (n=4) and high (n=2) concentrations of OIF/NGF in their ejaculates.

End point*	Male groups based on [OIF/NGF] in semen		
	Low (n=2)	Medium (n=4)	High (n=2)
OIF/NGF grouping (number of males)	Low (n=2)	Medium (n=4)	High (n=2)
Number of females bred	8	16	8
Follicle diameter at mating (mm)	8.8 ±0.55	8.2±0.33	9.2±0.72
Interval to ovulation (hr)	29.8 ± 1.4	28.2 ± 0.9	26.5 ± 1.5
Ovulation rate*	6/8 (75%)	15/16 (94%)	7/8 (88%)
Pregnancy rate**	4/8 (50%)	9/16 (56%)	3/7(44%)

* No statistical difference among groups for any end point

**The number of alpacas that ovulated or diagnosed pregnant divided by the number bred

Note that one alpaca was excluded from pregnancy rate due to illness.

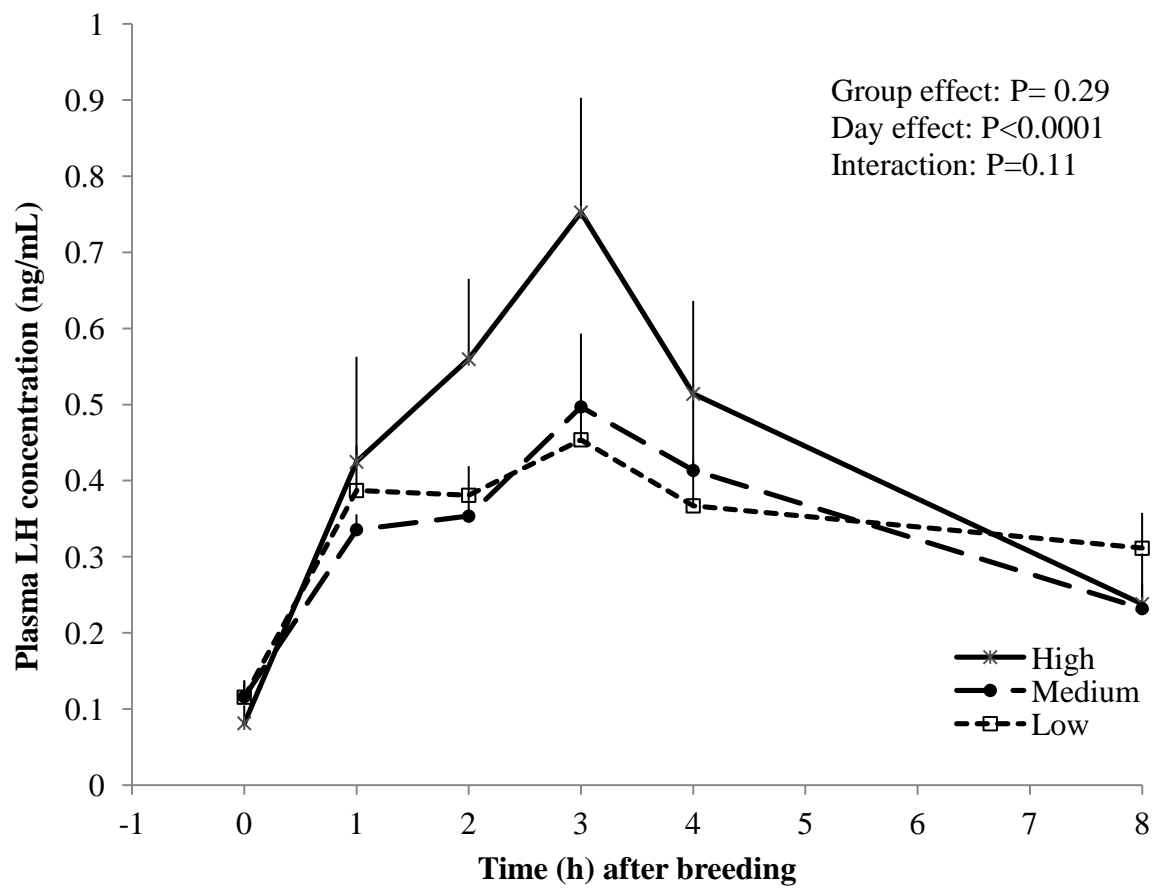


Figure 6. 1. Plasma LH concentrations (mean \pm SEM) in female alpacas that ovulated following mating with males categorized as having low (n=6 females), medium (n=14 females) or high (n=5 females) concentrations of OIF/NGF in their ejaculates.

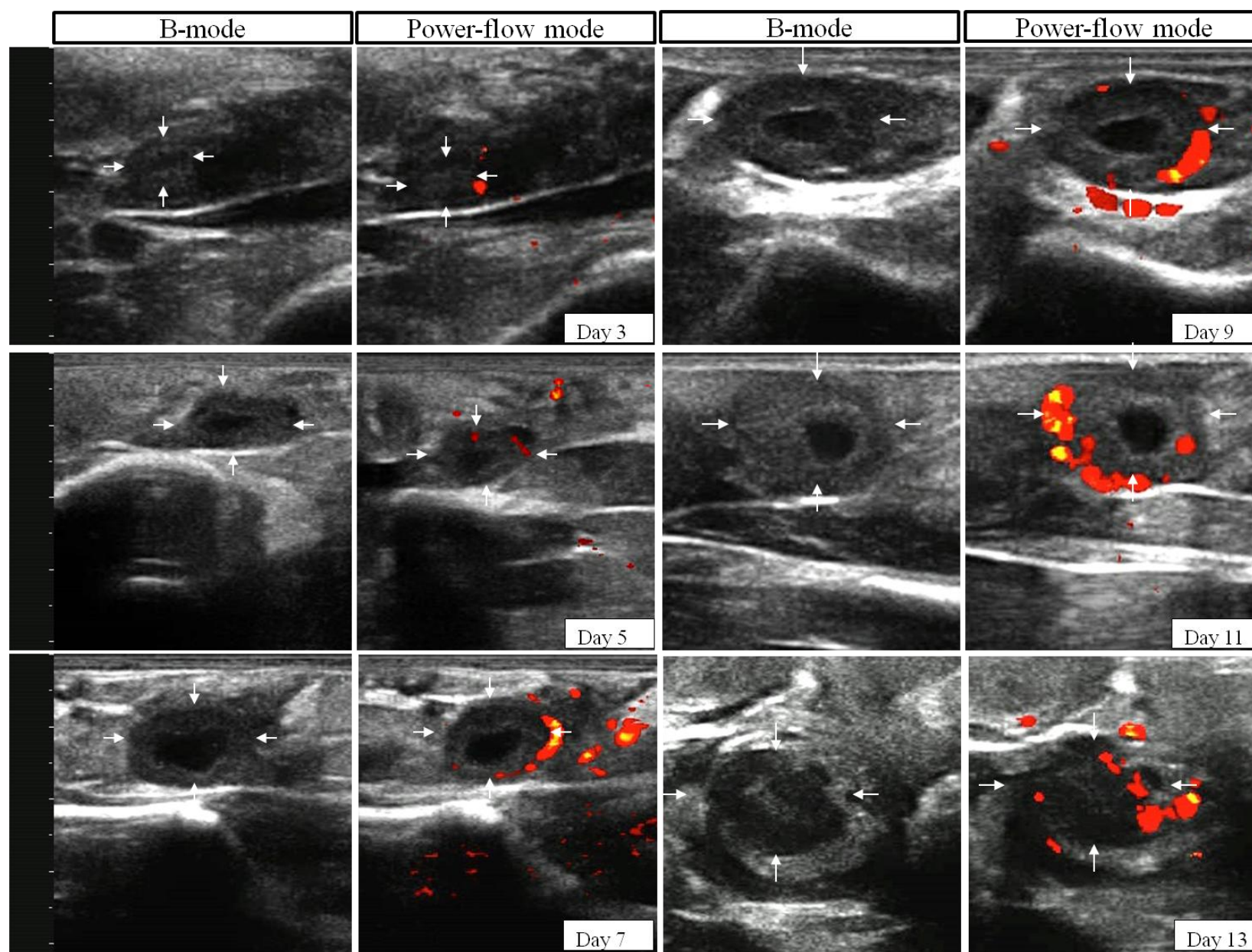


Figure 6. 2. B-mode and power flow Doppler images showing changes in the corpus luteum in a pregnant alpaca from Day 3 to Day 13 (Day 0 = day of breeding). White arrows in each image delineate the CL. The scale on the left is in 1 cm increments.

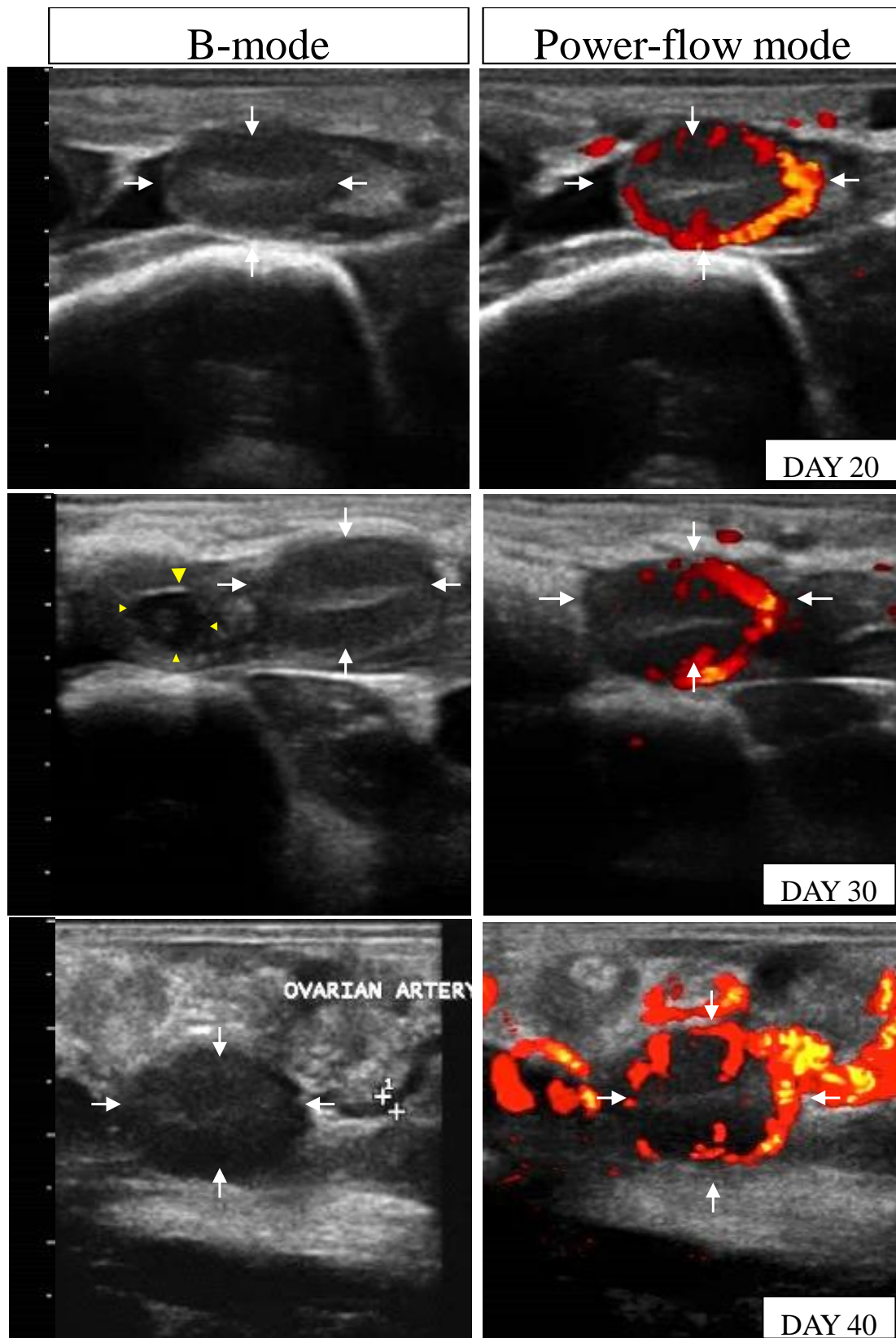


Figure 6. 3. B-mode and power flow Doppler images showing changes in the corpus luteum in a pregnant alpaca on Days 20, 30 and 40 (Day 0 = day of breeding). White arrows in each image delineate the CL. Yellow arrowheads demark the embryonic vesicle in the cross section of the left uterine horn (Day 30). The scale on the left is in 1 cm increments.

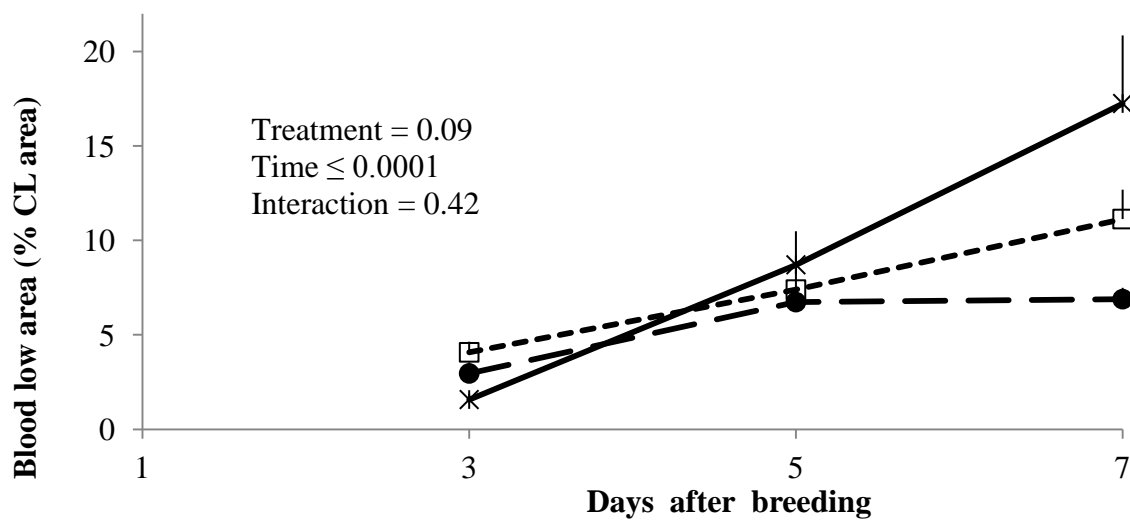
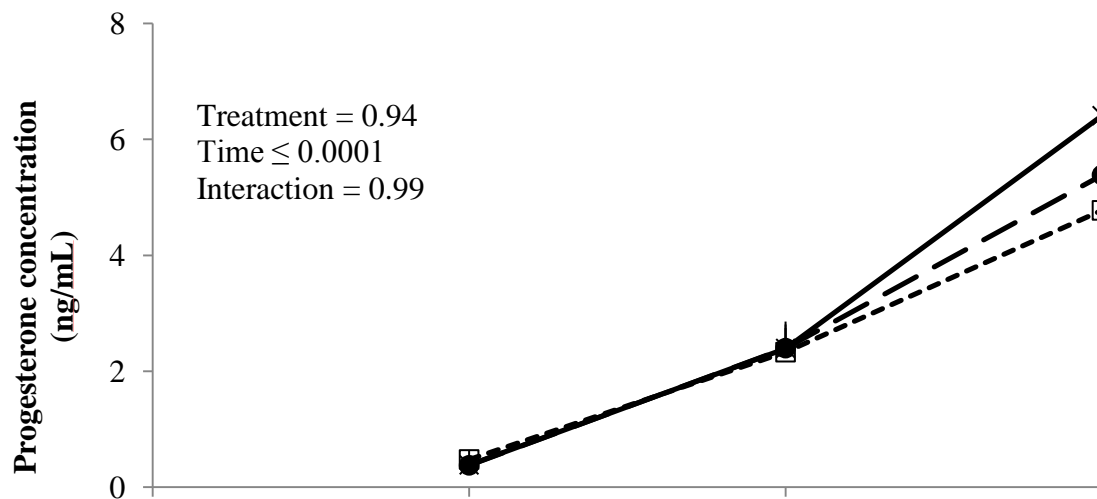
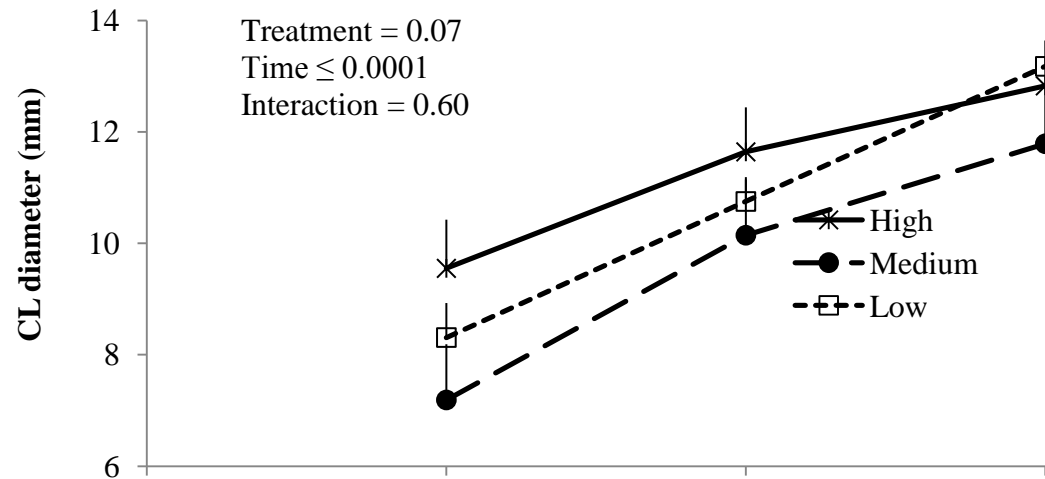


Figure 6. 4. CL diameter (top panel), circulating progesterone concentration (middle panel) and CL vasculature (bottom panel; Mean \pm SEM) in alpacas bred to males with low (n=6 females), medium (n=14 females), or high (n=5 females) concentrations of OIF/NGF in their ejaculates (Day 0 = day of breeding).

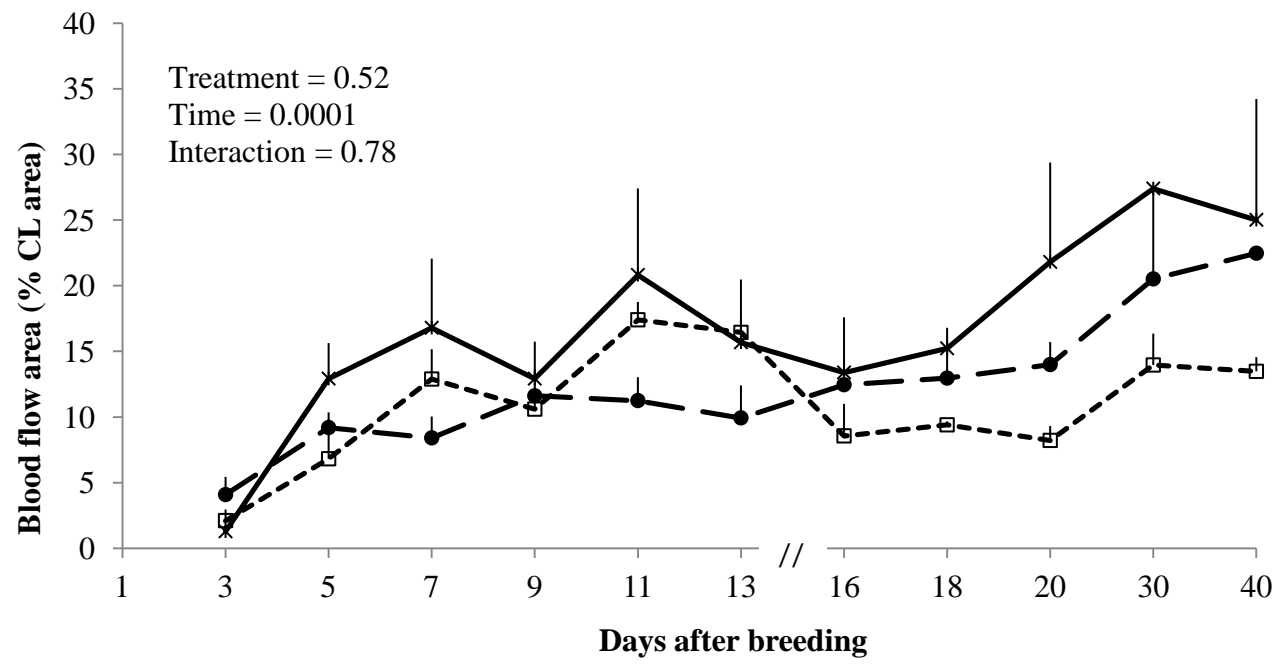
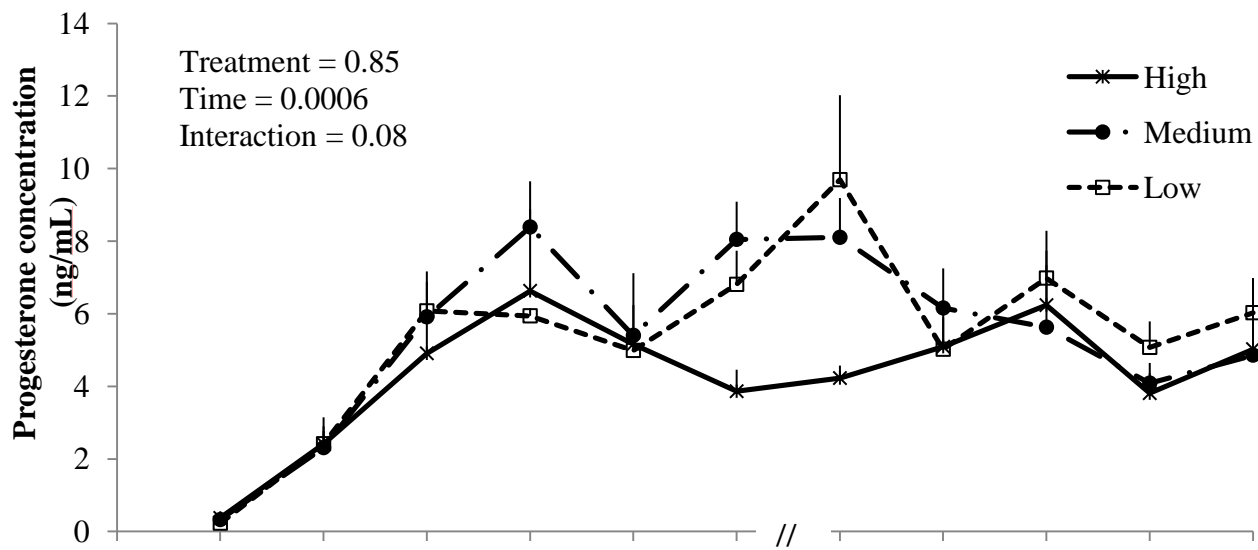


Figure 6. 5. Circulating plasma progesterone concentration (top panel) and CL blood flow area (bottom panel; Mean \pm SEM) in pregnant alpacas bred to males categorized as having low (n=4 females), medium (n=9 females), or high (n=3 females) concentrations of OIF/NGF in their ejaculates (Day 0 = day of breeding).

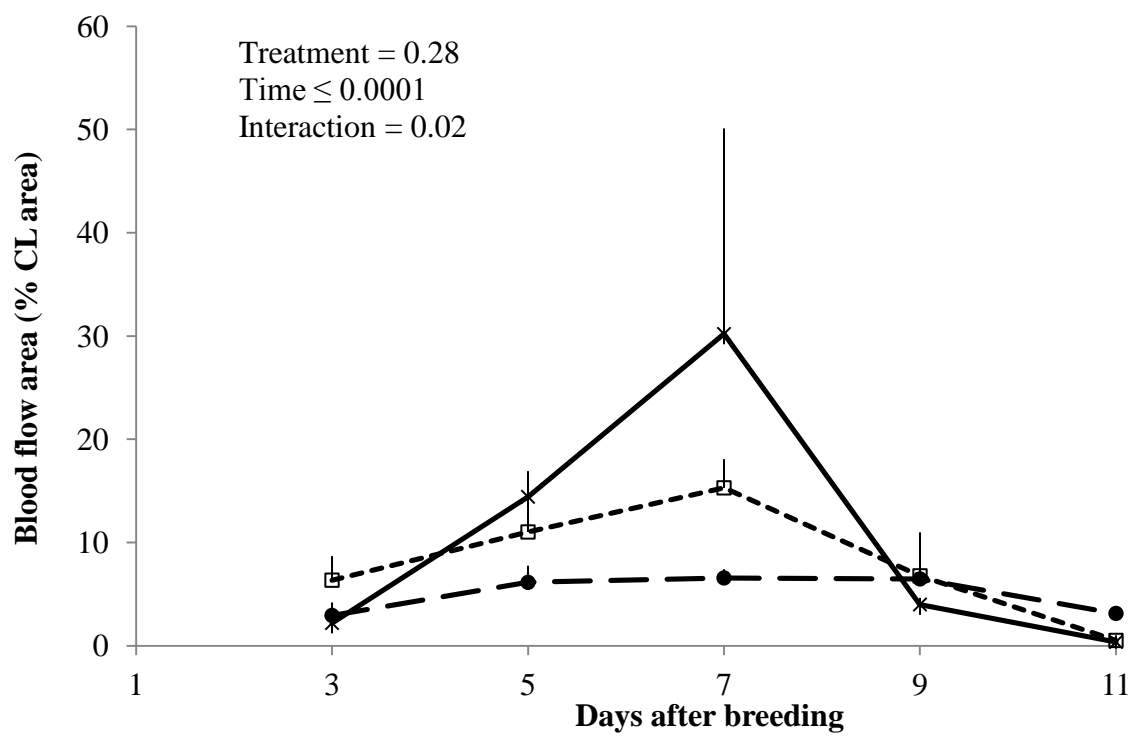
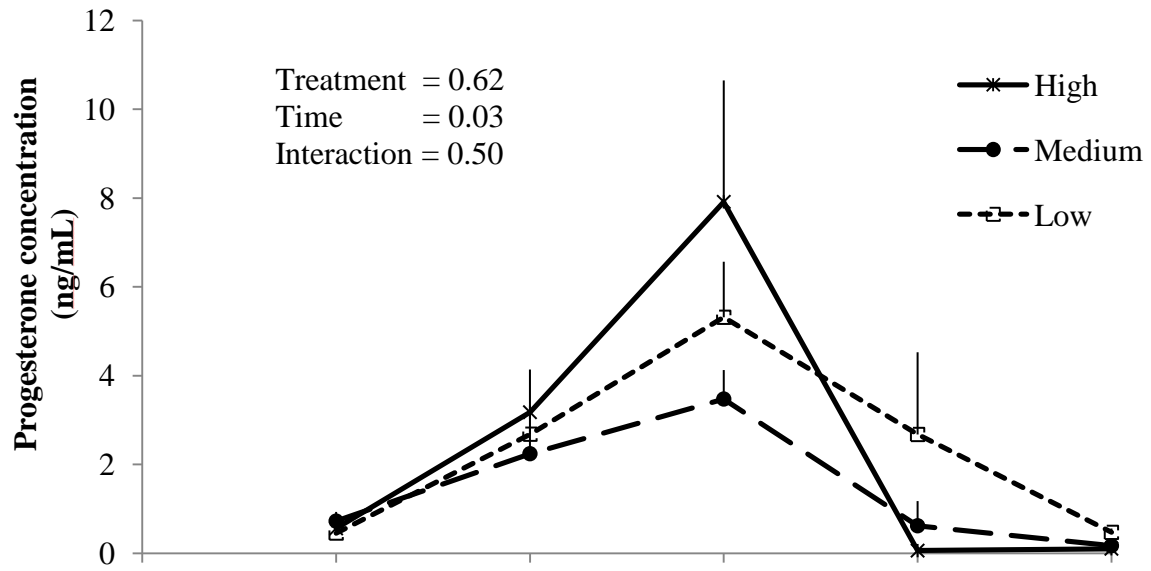


Figure 6. 6. Circulating plasma progesterone concentrations (top panel), blood flow area (bottom panel; mean \pm SEM) in alpacas that did not become pregnant after breeding to males with low (n=2 females), medium (n=5 females) or high (n=2 females) concentrations of OIF/NGF in their ejaculates (Day 0 = day of breeding).

* Blood flow area was greater in the high OIF/NGF group on Day 7 than in the medium OIF/NGF group (P<0.05).

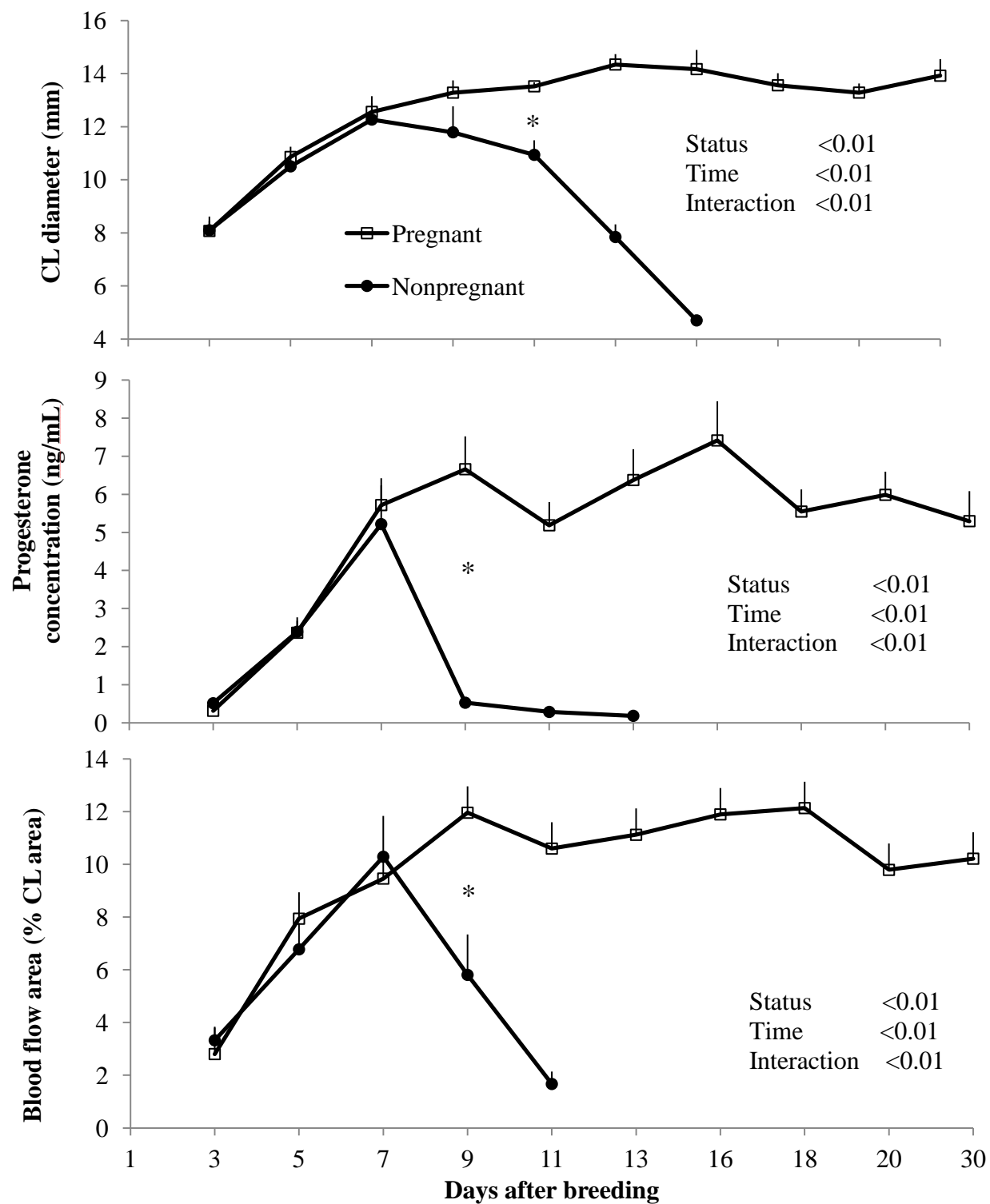


Figure 6. 7. CL diameter (top panel), circulating progesterone concentration (middle panel), blood flow area (bottom panel) in pregnant (n=16) and nonpregnant (n=9) alpacas (Mean±SEM; Day 0 = day of breeding).

* First day of significant difference between pregnant and nonpregnant animals

6.5 Discussion

We hypothesized that there is a positive relationship between the amount of OIF/NGF in the ejaculate of the male and the form and function of the CL in females to which they were mated. The trend across endpoints in the present study was consistent, in support of the hypothesis, though most differences were not statistically significant. Numerically, female alpacas bred to males in the high OIF/NGF group had a shorter interval to ovulation, larger pre-ovulatory LH surge, greater CL diameter and CL blood flow during early CL development, and higher plasma progesterone concentrations. While a luteotrophic effect of OIF/NGF has been reported previously when given by intramuscular administration [49, 175, 240, 254], the influence of differing quantities of OIF/NGF that occur naturally in male alpacas on luteogenesis in naturally mated females has not been previously examined. The consistency in results, and a better estimate of the degree of variance among factors in the present study, suggest that an increase in sample size will provide more definitive support for a cause-and-effect relationship between male OIF/NGF and female fertility.

In addition, we determined in retrospect that the amount of seminal OIF/NGF in the low group was likely not low enough to influence ovulation and CL development. Assuming a mean ejaculate volume for alpacas as 1.7 mL (Bogle, PhD thesis, Chapter 4), the total amount of OIF/NGF in the ejaculate of males in the low group was approximately 1.36 mg. This is more than the dose previously found to induce ovulation in 100% of treated alpacas [254] and more than 5 times the dose used to achieve a 90% ovulation rate in llamas [49]. Llamas given 125 µg - 500 µg of OIF/NGF intramuscularly, showed no difference in Day 8 CL diameter or lifespan [49]. Corpora lutea produced by intramuscular administration of OIF/NGF in llamas had a greater vascular area than those produced by administration of GnRH [175], but the dose-effect

of OIF/NGF among males in the present study may have been obscured by quantities of OIF/NGF that exceeded the minimum effective level. In this regard, all males used in the present study were used as sires in the breeding herd and had produced offspring in the past.

An increase in circulating concentrations of LH within the first hour of intramuscular treatment with OIF/NGF was taken as evidence for a systemic rather than local effect on ovarian activity [13, 48], and is consistent with the effect on LH secretion in the present study. However, NGF has also been shown to directly stimulate progesterone secretion from luteal cells *in vitro* [234], and it is possible that both a local and systemic effects of OIF/NGF are involved in luteogenesis. During copulation in camelids, the male ejaculates over a prolonged period (ranging from 3-65 minutes) and deposits semen directly into both uterine horns (reviewed in [333]). Based on anatomical descriptions of the utero-ovarian vasculature, both local and systemic luteolytic/trophic pathways may exist in camelids [201, 334]. A branch of the right uterine artery supplies the left horn and a corresponding major vein that originates from the left horn, terminates as a branch of the right uterine vein which allows rapid exchange of prostaglandin, metabolites, and possibly OIF/NGF into circulation between the ovary and the uterus [201]. As a consequence, the left uterine horn induces luteolysis in both ovaries while the right uterine horn may induce luteolysis only in the ipsilateral ovary [201, 334]. Nerve growth factor has been reported to have a direct local angiogenic effect on the ovary [331], cornea [319], muscle [321], skin [322], umbilical vein endothelial cells [320], circulating endothelial progenitor cells (CD34+) [335], and nervous tissue [336], providing rationale for the hypothesis that OIF/NGF induces angiogenesis in CL formation in camelids following mating.

Although we did not find a correlation between OIF/NGF abundance and CL vasculature in the present study, luteal flow area correlated with CL diameter and progesterone concentration

and was a useful technique for distinguishing between pregnancy and non-pregnancy. Luteal blood flow area increased from its first detection on Day 3 to a maximum on Day 7 in non-pregnant alpacas. A sharp decline in luteal blood flow area was detected on Day 9 concomitant with a sharp decline in plasma progesterone concentration – both of which preceded the first detectable decline in CL diameter on Day 11 in non-pregnant alpacas, similar to that previously reported [186]. In pregnant alpacas, luteal blood flow area was maximal by Day 9 and remained elevated, consistent with CL diameter and plasma progesterone concentrations in this and previous studies [10]). In general, the estimated percentage of the CL with blood flow signals in alpacas was lower than reported in cattle [313].

In conclusion, although results of endocrine and ultrasonographic endpoints tended to support the hypothesis that CL function is enhanced in female alpacas bred to males containing a greater quantity of OIF/NGF in their ejaculate, unequivocal support will require a larger number of females and selection of males representative of the minimum extreme in OIF/NGF in their ejaculate. Luteal blood flow measured by power flow Doppler, was strongly correlated with plasma progesterone concentration, and identified females that failed to become pregnant by Day 9; i.e., at the first significant decrease in plasma progesterone during luteolysis. Assessment of the CL by power flow Doppler may become a common tool for early pregnancy diagnosis.

7.0 GENERAL DISCUSSION

Other than a breeding trial, there currently is no method or biomarker to accurately predict fertility in male camelids. Because of the long gestational period (331-347 days in llamas [63] and 325-361 days in alpacas [73]), the identification of a biomarker and the ability to accurately measure its concentration would benefit camelid husbandry in terms of time and money. I investigated OIF/NGF in seminal plasma as a candidate biomarker for fertility in alpacas.

The effect of OIF/NGF on ovarian activity clearly documents its role as the principal stimulus for ovulation induction in camelids [38-41]. Because ovarian and endocrine responses to OIF/NGF are dependent on dosage, I hypothesized that the quantity of OIF/NGF in semen varied among males and this abundance arbitrates fertility in alpacas. The findings presented in this thesis were not sufficient to support my hypothesis. However, subsequent studies utilizing a different approach may be necessary to further test this hypothesis.

The cruxes of the studies presented herein involved measuring OIF/NGF in semen and observing the physiological response in females when there is a change OIF/NGF abundance. We know that the administration of OIF/NGF im or iv elicits the ovulatory cascade by triggering LH release and the level of response is dependent on the source of seminal plasma (camelid, cattle, rabbit, horse or pig) and the amount given [13, 39, 40, 42, 49, 52, 54, 55, 171, 293]. Although these studies demonstrate the biological activity of OIF/NGF, a perpetual criticism is the disregard of the "natural route" of administration, i.e. intromission or breeding. Hence, my overall objective was to demonstrate that similar to what has been published with im and iv routes of administration, breeding females to males containing increasing concentrations of OIF/NGF would result in greater ovarian response in female alpacas.

I believe that there are several possible explanations as to why we did not see a discernible difference among males with varying doses of OIF/NGF including high variation of OIF/NGF within ejaculates from the same male, excessive OIF/NGF in samples, low sample size and ejaculation differences between AV vs. breeding.

Determinants of male fertility are difficult to assess because of fluctuations found in semen parameters. Sperm concentration and morphology varies considerably with collection procedure, stress, season, climate nutrition [71, 84, 306, 337, 338]. High variability in OIF/NGF concentration, total abundance and other semen parameters were found within and among ejaculates. In both Chapters 3 and 4, a minimum of 3 ejaculates per male were collected to determine mean OIF/NGF abundance per each animal. Perhaps analyzing more ejaculates from the same male would have allowed a better representation of the total amount of OIF/NGF naturally occurring in each male.

The proportion that OIF/NGF comprises the camelid seminal plasma proteome was variable between the studies described in Chapters 3 and 4 (27% vs. 45%, respectively). Possible reasons for this difference include site of collection (Canada vs. Peru); season of collection (summer vs. rainy season); year of collection (2011 vs. 2012); frequency of collection (once vs. twice daily) and experience of males (companion animals vs. research breeding station). Animals were given the same relative training time but mounting efficiency was better in those animals from the research breeding station. As mentioned previously, froth volume was not taken into consideration when semen volume was calculated. This disregard may have contributed to error in total OIF/NGF quantification which would either over- or underestimate the total amount of OIF/NGF measured. Froth is commonly observed when semen is collected by AV[84]. However, we suspect that *in vivo*, this volume is liquefied. Artificial vagina inserted into a phantom mount

was the method chosen for semen collection because 1) we could mimic the sensory and olfactory stimuli associated with breeding and 2) evidence that electroejaculation could alter the seminal plasma proteome [276, 277]. Nevertheless, the possibility exists that the ejaculate collected by AV was not a good representation of what occurred during breeding. On the other hand, I observed that 90.2% of ejaculates collected by artificial vagina exceeded the dosage necessary for a 90% ovulation rate [49] and this percentage corresponds to the ovulation rate after breeding (Chapter 5).

The observation that seminal plasma or nerve growth factor administration (im) elicits an endocrine and ovulatory response in female camelids [13] suggests that the principle effect of NGF is systemic and through LH secretion. However, the different sources of OIF/NGF that could contribute or influence the ovulatory response needs further elucidation in camelids. Seminal plasma has also been associated with leukocyte recruitment to the site of ovulation in pigs [339, 340]. Inflammation has also been associated with elevated levels of NGF [341, 342] and the lengthy copulation duration and intrauterine deposition of the penis during intromission causes trauma to the vagina and endometrium [333]. Thus, there may be an endogenous source of NGF which contributes to the ovulatory response during mating. The ovary is also a source of NGF and trkA [343]. The expression of trkA appears dependent of LH: *in vitro* challenge of theca cells induced trkA expression after 8 hours [343]. Either blocking the effect of TrkA or immunoneutralization of NGF actions *in vivo* impaired ovulation in rats[344]. Furthermore, NGF stimulated both androgen and progesterone release in bovine thecal cells [325].

The secretion of LH in response to OIF/ NGF challenge has been examined in camelids by *in vivo* and *in vitro* bioassays. The role of NGF on camelid follicular dynamics and FSH secretion has not been investigated. Overwhelming evidence in humans and rodents demonstrate

that NGF and TrkA are important factors in follicular development and ovulation [231, 232, 325, 343, 344]. NGF increases the expression of FSH receptors in antral cells [233, 324, 325] and elevated levels of NGF have been associated with polycystic ovarian syndrome [227, 229]. One problem in assessing the effect of NGF on ovarian follicular dynamics in camelids in the early follicular phase is the presence of an ovulatory follicle in either ovary [130]. However, follicle ablation followed by OIF/NGF treatment could be used to determine whether NGF would induce FSH secretion, hasten wave emergence and recruit more ovarian follicles which could be used in superstimulation protocols.

One weakness in the experimental design was that I was unable to measure OIF/NGF prior to breeding trials. On one hand, this ensured a complete blind experiment where no one had any knowledge OIF/NGF concentration or mating history. However, in light of this, 8 males were chosen at random for breeding trials and then arbitrarily divided into low, medium or high OIF/NGF groups. The ability to measure OIF/NGF prior to breeding would have allowed better selection of males that either had less intra-variation within ejaculations and males that had OIF/NGF protein levels below the threshold value of 250 μg .

The correlation between sperm morphology and fertility has not been explored in camelids as it has in cattle [337, 345-347]. In Chapter 5, I observed that pregnancy rates in alpacas were positively correlated with sperm concentration and viability. Factors in semen that were associated with infertility or nonpregnancy were semen pH, and sperm morphologies including midpiece and acrosome defects. Correspondingly, the male that produced the highest ovulation rates had low sperm concentration which is most likely the reason for low pregnancy rates. Sperm head morphology (which includes acrosome defects) was the most common defect observed among ejaculates which indicates disturbances arose during spermatogenesis and not

during epididymal transport. The fertilization capability of sperm with varying defects need further investigation.

Mounting behaviour was not critically assessed in this experiment. However, the male that showed unrestrained sexual interest to females, also had poor mounting efficiency, frequent interrupted matings, low ovulation rates and low pregnancy rates. This finding was interesting because it further demonstrated that other mating stimuli such as male humming, tactile stroking of the female hindquarters and scent was insufficient to induce ovulation.

The relationship between sexual behaviour and neural circuits within the central nervous system is one that has been investigated in several species. Vaginal stimulation in women activated various regions of the brain including the hypothalamic paraventricular nuclei [348, 349]. In women, neuroimaging showed the female orgasm was associated with influx of blood supply to the pituitary gland [350]. Plasma concentrations of pituitary hormones, LH, oxytocin and prolactin, have also been shown to increase in association with intercourse and orgasm [351-354]. Vaginal stimulation in the ewe was found to significantly increase oxytocin release in the mediobasal hypothalamus when ewes were sexually receptive. When ewes were under the influence of progesterone or were not sexually receptive, vaginal stimulation had no effect on oxytocin release [355]. In contrast, plasma concentrations of cortisol, FSH, β -endorphin, progesterone and estradiol were unaffected by orgasm [354]. To my knowledge, LH is the only hormone that has been measured in camelids in response to mating, however strong evidence suggests that other pituitary hormones such as oxytocin and prolactin should be investigated.

To my knowledge, this is the first report that the site of storage and secretion of nerve growth factor is the prostate gland in camelids (Chapter 3). OIF/NGF was stored in the glandular

epithelium as secretory vesicles and observed in the lumen of acini and blood vessels. Correspondingly, OIF/NGF was greater in the ejaculates of males that had larger prostate glands. Because OIF/NGF found in camelid semen appears to come from one gland, it would be interesting to ablate the prostate and compare OIF/NGF concentration, ovulation and pregnancy rates before and after its removal from the same male. This design would provide the advantage of eliminating inter male variation.

The observation of NGF in circulation suggests a role in other reproductive tissue. In rodents, both trkA and p75 receptors have been localized in germ cells, Sertoli cells and accessory genital glands, including seminal vesicles, prostate and coagulating gland [288]. A relationships between endocrine activity and behaviour has also been made in a variety of mammals. In humans and rodents, NGF concentration in circulation increases in relation to stress [263, 267, 287, 356]. An intriguing observation was that NGF in circulation was higher in aggressive male mice and the hypothalamus was one region where altered NGF detection was related to this behaviour [209, 268, 286]. Intravenous injection of NGF into rats produced a dose dependent increase in circulating concentrations of adrenocorticotropic (ACTH) and corticosterone [357, 358]. While cortisol levels increased in men undergoing acute stress, NGF concentration in circulation remained unchanged [359].

Although male behaviour was not critically measured in the context of this thesis, there was no relationship between testosterone concentration and total NGF content found in the ejaculate. A relationship between testosterone concentration and mating or aggression could not be inferred in the context of this thesis as only one blood sample was collected. Nevertheless, we illustrate an additional source for NGF secretion that contributes to its levels detected in circulation which is rational to investigate the role of NGF in the hypothalamic-pituitary-testis.

To my knowledge the effect of exogenous OIF/NGF in male camelids has not been investigated. However, the secretion of LH in response to OIF/NGF or seminal plasma observed in females [45, 48, 52, 54, 239] may be mimicked in males. Pituitary LH stimulated testosterone secretion by Leydig cells, and FSH optimizes testosterone concentration in the seminiferous tubules by increasing the production of androgen binding protein in the Sertoli cells [360-363]. Treatment of Holstein bulls with GnRH resulted in an increase in scrotal surface temperature, LH and testosterone but did not increase sperm output [364, 365]. In addition, immunization against GnRH in male camels showed a reduction in testosterone concentration, libido and acrosome morphology but did not influence testicular volume [366]. Although testes size was not a reliable measurement of sperm output in pubertal cattle [367] or alpacas (Chapter 5), the endocrine response to exogenous treatment of OIF/NGF and whether changes are reflected in spermatogenesis needs to be determined.

An association between OIF/NGF quantity at the time of breeding and CL luteogenesis could not be determined in this study (Chapter 6). However, the tendency for higher CL tissue vasculature in the OIF/NGF group suggests that repeating the experiment with a greater number of animals is needed. An interesting finding was that nonpregnant alpacas could be detected earlier from pregnant ones using power Doppler to measure CL vasculature.

To summarize, the information presented in this thesis contributes to our understanding of the role of nerve growth factor in camelid physiology. To our knowledge, this body of work includes the first account describing the prostate gland as the primary source of OIF/NGF secretion in the camelid ejaculate (Chapter 3); the quantity of OIF/NGF in alpaca seminal plasma (Chapters 3 and 4); semen parameters associated with ovulation and pregnancy rates in alpacas

(Chapter 5); the use of power Doppler to measure CL tissue vasculature for early pregnancy detection in alpacas (Chapter 6).

8.0 CHAPTER CONCLUSIONS

CHAPTER 3

Principle observations:

- OIF/NGF was found in the connective tissue of all organs
- OIF/NGF is stored and produced in the prostate gland of llamas
- OIF/NGF is found in the vesicular and ampullar glands of cattle
- OIF/NGF is found in the vesicular and ampullar glands of bison
- OIF/NGF is found in the ampullar gland of elk
- OIF/NGF is found in the prostate gland of white tail deer
- OIF/NGF represented approximately 27% of the alpaca proteome in seminal plasma
- OIF/NGF represented <1% of the proteome found in bull seminal plasma
- Radioimmunoassay developed can detect OIF/NGF in seminal plasma from bull, alpaca and llama seminal plasma
- OIF/NGF was not detected in llama blood or seminal plasma from stallions and boars
- No cross reaction of the NGF antibody was observed with NT4 and BDNF

CHAPTER 4

Principle observations:

- OIF/NGF concentration but not total OIF/NGF abundance was associated with sperm concentration

- Positive correlation was observed between total OIF/NGF per ejaculate and total semen volume and prostate area

CHAPTER 5

Principle observations:

- Males with higher quantities of OIF/NGF in their ejaculates did not have better ovulation or pregnancy rates
- Pregnancy rates were related to sperm concentration, morphology, semen pH and female age

CHAPTER 6

Principle observations:

- Power Doppler colour pixels were an accurate assessment of progesterone concentration and CL viability
- The use of power Doppler colour pixel measurement detected pregnancy earlier than with CL measurement by B-mode ultrasound
- Males with greater OIF/NGF abundance in semen did not produce more luteogenic CLs

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